THE USE OF CHILLING IN THE ANALYSIS OF THE FIRST STAGES OF INFECTION BY INFLUENZA VIRUS

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In our work on the action of ribonuclease on the multiplication of influenza virus in the de-embryonated egg (Burnet, Lind and Perry, 1957) some reference has been made to the use of chilling to allow more precise demarcation of phases in the process. We are here concerned to describe the influence of manipulations in the cold (0° C.) on the result of double infections, using our standard M+ and WS— strains. By titrating the yield for haemagglutinin in anti-MEL and anti-WS serum as well as in saline, information can be obtained in regard to the relative amounts of each virus and of the extent to which double neutralization occurs.

It will be shown that virus added first in the cold is at a definite disadvantage over that added second, but if a period of warming (at 37° C.) is interpolated between the two inoculations of virus the situation is rapidly reversed. Very little reproduction of the second virus can be demonstrated if the preparation infected with the first virus in the cold is brought to 37° C. for 30 minutes before adding the second virus. The two initial stages of the process of infection so defined are probably identical with those recently reported, using a wholly different approach, by Ishida and Ackermann (1956).

MATERIALS AND METHODS.

Virus strains. In almost all work the strains M+ and WS— were used. These were originally isolated as recombinants from the standard strains MEL and WSE (Burnet and Lind, 1952) and have been used in many investigations primarily because of the high yield of MEL recombinants which is obtained. For each experiment fresh stocks of infective fluid were prepared.

De-embryonated eggs (DEE) were prepared from 14-16 day chick embryos by the method previously described (Burnet and Lind, 1954). All washing was done with Ringer's solution and the medium (GPSR) used during experimental manipulations consisted of Ringer's solution containing 0·1 p.c. glucose and penicillin-streptomycin (40 units + 50 μg. per ml. respectively).

Rotors. The rotating turntable on which eggs are incubated with their long axes at an angle of 15° to the horizontal has been described previously (Burnet and Lind, 1954). This is enclosed in a cabinet maintained at 37° C. For the present work a similar rotor was placed in a cold room held at 0-1° C. These are referred to as warm and cold rotors respectively.

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General form of experiments. In all experiments virus diluted to a level equivalent to 10 or 20 haemagglutinating doses (AD) was added to the DEE in 5 ml. amounts and allowed 1 hour (warm) or 2 hours (cold) for adsorption to the allantoic cells. The inoculum was removed, the DEE washed three times with Ringer's solution (at 0° C. or at 37° C. according to the needs of the experiment) and, after any requisite manipulations, refilled with GPSR and returned to the warm rotor. Harvests of fluid were removed for haemagglutinin (HA) titrations at appropriate times. For reasons of convenience most results are from harvests taken after overnight incubation. With the large inocula used there is no significant difference between 15 and 22 hour yields.

The harvested fluid was titrated using a "pattern" method in "perspex" trays with serial dilutions: (a) in saline; (b) in 1 : 200 dilution of anti-MEL (M) serum, and (c) in 1 : 200 anti-WS serum. Both antisera had been adsorbed with the heterologous strain by the method of Edney and Lim (1954) and were fully specific, neutralizing about 100AD of the homologous virus and having no significant action on the heterologous one.

The results so obtained give information (a) of the total HA produced, (b) of the relative dominance of MEL (M) or WS serotypes in the harvest, and (c) of the proportion of doubly neutralized virus. In most tables the results shown are the average values from 2 to 4 DEE of the HA titres obtained by titration in saline, of the relative dominance (R.D.) of WS serotype over M serotype and of the extent of double neutralization (DN).

The procedure used in calculating the results may be illustrated in Table 1 which gives the data from one of the control groups in an experiment to be described later (see Table 2). In this group a mixture in equal parts of M+ 40AD and WS– 20AD was prepared, the final concentration of each virus being half that stated. This was added warm to 4 DEE and maintained on the warm rotor at 37° C. for 1 hour. The DEE were then washed and the fluids harvested after 18 hours at 37° C.

TABLE 1.

Yield from M+/WS– mixture maintained throughout at 37° C. and harvested overnight.

HA titres			Relative Dominance (RD) $\frac{WS-}{M+}$	Double Neutralization (DN)
Saline	Anti-M	Anti-WS		
110	5(5)	25(23)	$\frac{9{\cdot}5}{9{\cdot}2}$ 1·0	18·7 p.c.
180	30(16)	10(5)		
90	8(9)	6(7)		
350	30(8)	8(2)		

Figures in brackets show percentage of HA titre in saline, the means for the group being Anti-M 9·5 p.c., Anti-WS 9·2 p.c.

The HA titre in saline is taken as a basis and for each fluid the titres in anti-M and anti-WS sera are converted to a percentage of the saline titre. These percentage values are averaged and expressed as a fraction.

$$\frac{\text{P.c. titre in anti-M}}{\text{P.c. titre in anti-WS}} = \text{R.D.}$$

The titre in anti-M is taken as a measure of virus of WS serotype and in anti-WS of the M

serotype, so that the fraction is an expression of the relative dominance (R.D.) of WS over M serotypes.

Double neutralization is indicated by summing the percentage values in the two antisera. The smaller the value the more extensive the double neutralization and on the orthodox interpretation the larger the proportion of virus particles carrying a mosaic of M and WS components. In the example shown, approximately 81 p.c. of the HA is shown as neutralizable by both antisera. In practice any value over 50 p.c. for the combined percentages is taken as indicating that no significant double neutralization can be postulated.

EXPERIMENTAL.

The relative lack of effect of chilling on the yield of haemagglutinin. When virus is added to DEE at 0° C., it is found that the haemagglutinin disappears almost completely within 2 hours, presumably by adsorption to the free surface of the allantoic cells. There is some variation from one preparation to another but to a fairly close approximation an initial HA titre of 20-25 is reduced along a logarithmic course to about 2, after 2 hours' rotation in the cold. Fig. 1 gives the results of haemagglutinin titrations on 4 DEE inoculated with the standard M+/WS— mixture and with the two individual strains.

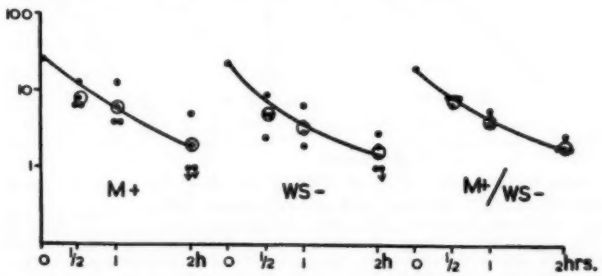

Fig. 1. Adsorption of haemagglutinin (M+, WS— and equal mixture of the two strains) in DEE rotated at 0° C.
Ordinates: HA titres of samples taken at the time shown in hours: logarithmic scale. Circles indicate mean titres, dots individual values.

Apart from a slight slowing and sometimes a minor depression in yield of haemagglutinin, there is no alteration in the course of virus production at 37° C. if at any part of the sequence a period of 16-20 hours at 0° C. is interposed. In Fig. 2 are shown first the normal sequence of HA titres obtained in our standard procedure in the warm rotor. In the other graphs a period of 16-20 hours in the cold was interposed.

A. Before any addition of virus.
B. Before and during the adsorption of virus.
C. Immediately after virus had been adsorbed.
D. After virus had been adsorbed, the DEE washed and incubated for one hour at 37° C.

In this experiment strain MEL was used. It will be seen that the only significant deviation occurs when the adsorption of virus takes place at 0° C. Here there is a rather marked slowing of the initial liberation of haemagglutinin.

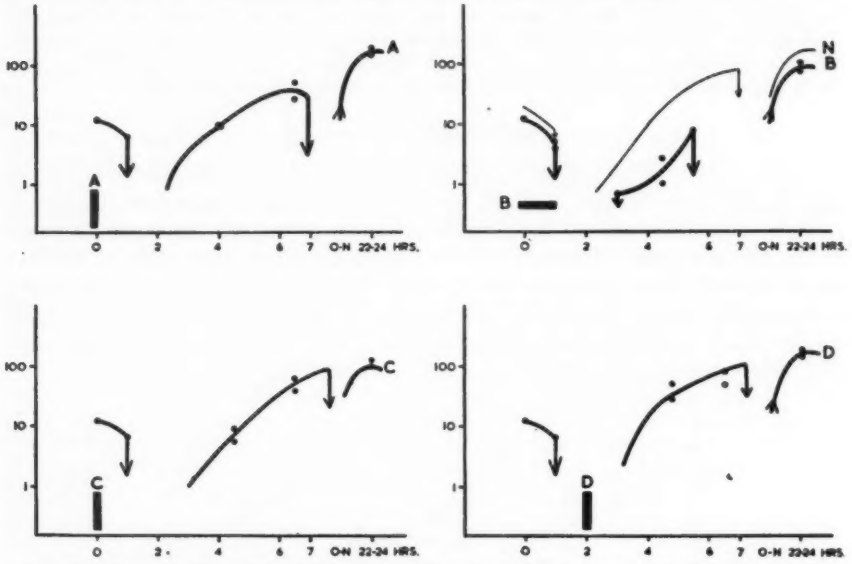

Fig. 2. To show the minimal effect of chilling for 16-20 hours at 0° C. on subsequent liberation of virus from DEE infected with 20 AD strain MEL.

In each graph, ordinates represent HA titre (logarithmic scale). The time scale in hours concerns only the period when the preparation was held in the warm rotor. Heavy bars are shown at the periods where an overnight stay at 0° C. was interpolated. After 7 hours (or its equivalent) at 37° C. the DEE were emptied and refilled with fresh medium. After overnight (O–N) incubation the final titration was made 22-24 hours after initiation of the experiment.

N. Standard course at 37° C. throughout.
A. 0° C. before inoculation.
B. 0° C. before and during adsorption of virus.
C. 0° C. immediately after virus had been adsorbed at 37° C.
D. 0° C. after virus had been adsorbed, the DEE washed and a further period of 1 hour at 37° C. had been given.

Relative dominance of the two serotypes in relation to times of inoculation. When one virus is added to DEE on the cold rotor and the second subsequently added in the cold, allowed to adsorb, and the DEE then incubated at 37° C., the virus added *second* gains a distinct advantage. This is more strikingly shown when M+ is the virus first added than when WS— has precedence. When M+ and WS— are added together in equal amount in the cold, WS— usually dominates in the final yield and in some experiments 2 parts of M+ to 1 of WS— were used to obtain a more balanced yield. In the experiment shown in Table 2 the dominance of WS— is unusually high, but the increase in WS— dominance when M+ is given first and the decrease when WS— is given first is evident.

TABLE 2.

Effect of time of addition of the two strains on the nature of the haemagglutinin yield.

Procedure	Temperature	HA titres	Relative Dominance WS−/M+	DN (p.c.)
M+ 2 hours: WS− 2 hours	Cold	152	6·3	40·5
WS− 2 hours: M+ 2 hours	Cold	167	2·6	25
M+/WS− together 2 hours	Cold	145	4·5	25·7
M+/WS− together 1 hour	Warm	182	1·0	18·7
M+ alone	Warm	240	<0·01	83
WS− alone	Warm	160	>100	100

In connection with various experiments, including those to be mentioned in the next section, a considerable number of values for the relative dominance (R.D.) of the viruses was obtained. In view of the considerable variation from one experiment to another in the R.D. shown by the control in which the viruses were added simultaneously in the cold, we have recalculated the result to show the *change* in the R.D. when one or the other virus was added first in the cold. The R.D. values in Table 2 are 4·5 for the control mixture, 6·3 when M+ was added first and 2·6 when WS— was added first. The change then can be expressed as: M+ first 6·3/4·5 (1·4), WS— first 2·6/4·5 (0·6). Using this method the change in R.D. observed over the whole series was as shown in Table 3. It will be clear from the mean values obtained that much more striking change is obtained with M+ as the first virus than with WS—.

TABLE 3.

Change in relative dominance in relation to which virus was first added in the cold.

M+ first			WS− first		
1·4	3·0	0·8	0·6	0·7	0·9
1·3	2·0	5·6	0·7	1·0	0·8
4·1			1·2		
Mean 2·60			Mean 0·8		

The influence of RDE on the phenomenon. One possible interpretation of these findings is that the virus first presented to the cells, especially if it is M+, becomes progressively more firmly attached to available receptors. On rewarming, it takes longer to break down a large number of such unions by the virus neuraminidase (Gottschalk, 1957) than to break down a smaller number. There is no direct evidence that this occurs, but it is very much in accord with the interpretation derived from various types of evidence which was figured diagrammatically by Burnet (1955). In this the mucoprotein of the cell surface is represented as bearing many prosthetic groups which in the cold will serve as points of adsorption and on warming as substrates of the virus enzyme.

An experimental approach to test this hypothesis is to show whether pretreatment with RDE, which should greatly reduce the number of receptors and hence the development of a progressively firmer union in the cold, will annul the change produced by using M+ first.

The experiments were carried out as follows, either 3 or 4 DEE being used to obtain each set of results. The DEE were first treated on the rotor at 37° C. for 30 minutes with 5 ml. of RDE (having a titre of 200 units) prepared in GPSR, or with GPSR alone. The eggs were then emptied, washed with cold Ringer and taken to a cold room at 0° C. Here one set (A) from each group received 20AD of the first virus and the eggs were than placed on the cold rotor for 2 hours. The other set (B) from each group received cold GPSR only for the same period. At the conclusion of the 2 hours at 0° C. all DEE were emptied, set A received 20AD of the second virus, set B was given 5 ml. of an equal mixture 20AD of the two viruses. After another 2 hours at 0° C., all DEE were washed 3 times with Ringer, refilled with 5 ml. of warm GPSR and incubated on the 37° C. rotor overnight.

Three experiments are shown in Table 4 in order to indicate the considerable degree of variability observed in these experiments from day to day. In the third experiment A^2A^3, M+ given first failed to show the usual increased dominance of WS— but in the presence of RDE M+ became much more dominant.

TABLE 4.

Effect of pretreatment with RDE on yield from DEE inoculated at 0°C.

Procedure		No RDE		+RDE	
		HA	R.D.*	HA	R.D.*
A	M+ before WS−	170	7·1	52	0·5
B	M+/WS− control	160	4·1	31	0·9
A^1	WS− before M+	100	2·2	50	1·5
B^1	M+/WS− control	82	8·0	40	1·0
A^2	M+ before WS−	270	1·0	47	0·2
A^3	WS− before M+	200	1·2	55	0·8
B^{2-3}	M+/WS− control	110	1·6	58	0·18
	M+ alone	50	—	177	
	WS− alone	117	—	10	

Each set of values for HA and RD is the mean of 4 DEE.
* R.D. = Relative Dominance.

The outstanding feature of the results obtained was the much greater effect on WS— than on M+. All harvests showed a sharp reduction in the dominance of WS serotype when the DEE had been pretreated with RDE. The very marked effect on WS— made it impossible to be certain that the reversal observed had any more general significance. However, as far as the results have

gone, they are consistent with the hypothesis that the effect is related to the number of receptors available and the firmness with which they can link to the virus. It will be noticed that the change produced by RDE when WS— is given before M+ is much less than in the M+ before WS— experiments. In other words, there is a distinct trend for the advantage to be transferred to the virus first presented.

Development of dominance at 37° *C.* Preliminary experiments had established that if the second virus was added after the first had been in the DEE for an hour at 37° C., the yield obtained was heavily dominated by the first virus. From the point of view of obtaining information about the early stages of intracellular growth it was therefore of considerable interest to determine at what time virus added in the cold would be able to establish dominance over a second virus added after a period during which the DEE were incubated at 37° C.

The technical procedure was to allow adsorption of virus for 2 hours in the cold. The eggs were washed once with chilled Ringer's solution then filled with warm GPSR and placed on the 37° C. rotor for the desired time. The DEE were then emptied, washed once with warm Ringer, inoculated with 5 ml. of the second virus, previously chilled to 0° C., and returned to the cold room for approximately 2 hours. The whole series were then washed, refilled with warm Ringer and left overnight on the warm rotor. Two variations in treatment subsequent to the warm period gave results of the same general character. These are indicated in Table 6 by the symbol, A, when the second virus was added warm without any second period of chilling, B, when 2 hours at 0° C. with GPSR alone preceded inoculation of warmed virus for one hour at 37° C., followed, after washing, by the standard overnight period of incubation at 37° C.

Table 5 gives the findings of one experiment in detail. Here it seems that after 10 minutes at 37° C. the disadvantage of first presentation has been over-

TABLE 5.

Development of dominance of first added virus.

First virus in cold	Period at 37°C.	Second virus	HA	Relative Dominance WS/M	DN (p.c.)
M+	0 min.	WS−	155	8·1	32·5
M+	10 min.	WS−	105	0·7	54
M+	30 min.	WS−	175	1·4	13
M+	60 min.	WS−	70	0·17	91
M+	0 min.	Nil	90		
Nil	—	WS−	120		
WS−	0 min.	M+	210	0·7	19
WS−	10 min.	M+	190	1·0	21
WS−	60 min.	M+	200	5·0	65

come by M+, but its full dominance is developed between 30 and 60 minutes. To present results compactly the change in dominance can be expressed in similar fashion to that used for the "cold" results (see Table 3). Here, however, the basis is the value of R.D. obtained when the first virus was followed, after 2 hours in the cold, by the second without any intervening warm period. This is given a value of 1·0 and the other R.D. values correspondingly adjusted. The figures in Table 6 are calculated in this way to show the combined results of

TABLE 6.

Change in dominance with period of intermediate exposure to 37°C.

Period at 37° C.	M+ first						
0	1·0ϕ (standard)						
10 min.	0·09	0·15					0·9
30 min.	0·18	0·035	0·031[A]	0·046	0·011[B]	0·012	0·61
60 min.	0·023	0·026					0·046
	WS— first						
0	1·0 (standard)						
10 min.	1·4						
30 min.	30	20·5[A]	6·5	19·5[B]			
60 min.	7·1						

A, B. Results with modified techniques described in the text.

several experiments. The results indicate a moderate degree of variability, but in the case of M+ first there is a highly significant change in two of the three results at 10′; at 30′ dominance of M+ is evident in six out of the seven and nearly complete in five. With WS— first all showed virtually complete dominance of WS. virus at 30 minutes.

DISCUSSION.

Perhaps because of its function as a receptacle for waste metabolites the allantoic cavity is bounded by cells which are extremely tolerant of non-physiological conditions. In the DEE, influenza virus will multiply freely when the bathing fluid is simple physiological saline. It was therefore not unexpected to find that even prolonged chilling to 0° C. had very little effect on the subsequent capacity of the preparation to allow influenza virus multiplication.

We have exploited the potentialities of chilling primarily in an attempt at a closer analysis of the action of RNase on the process, but the present experiments throw some light on the first stages of infection in the cell.

It is generally accepted that adsorption of virus particle to the cell requires, or is at least facilitated by, union of the enzymic-adsorption groups of the virus to mucoprotein receptors on the cell surface. Pretreatment with RDE diminishes the number of receptors available and diminishes the frequency with which infection is initiated.

It was already known from the work of Ishida and Ackermann (1956) that effective union of virus and allantoic cell could take place in the cold. Our findings in regard to the disadvantage of being first adsorbed indicate that the virus taken up at the cell surface becomes progressively more firmly attached while the low temperature is maintained. Virus subsequently added in the cold is less firmly adsorbed. This may be due both to the shorter time available and to the occupation of the most accessible or avid cell receptors by the first coming virus. When the system is warmed to 37° C., virus, in order to pass to the next stage of the process of infection, must liberate itself from its primary union with the cell receptors presumably by enzymic action. The second virus, having less numerous and weaker bonds to break, is therefore able to gain a dominance over the one first present in the competition for the host cell components necessary for the next stage of multiplication. It is of interest that the phenomenon is not symmetrical as regards M+ and WS—. When M+ is first presented to the cells the advantage conferred on WS— is significantly more than when the positions are reversed.

The use of RDE before virus is added removes this effect completely and allows the first added virus to compete on equal terms with the second. When the available, and particularly the most accessible, receptors are destroyed by RDE, virus receptor unions are rare and easily broken by subsequent enzymic action by the virus itself. In addition, a smaller proportion of cells is infected with consequent lowering of the final HA yield and fewer cells suffer double infection, so that there is less evidence of double neutralization in the product. Pretreatment with RDE inhibits infection by WS— more than by M+, irrespective of whether the viruses are tested alone, in sequence or together. This is completely in line with the asymmetry of the time effect observed in the absence of RDE and greatly strengthens the interpretation that has been offered.

A limited period at 37° C. (less than 30 minutes) is sufficient to remove the disadvantage of prior attachment and allow a virtual monopoly of eventual yield to the first virus. Attempts to make a more precise estimate of the time required for this change were hindered by the considerable variability from one experiment to another. In every experiment the dominance of the first virus was complete if 60 minutes was given at 37° C.; this held for most experiments giving 30 minutes at 37° C., but there were two exceptions in the nine results obtained. With 10 minutes at 37° C. two experiments in which M+ was given first showed that the two viruses had reached an approximate balance.

We can deduce therefore that in the system being used it takes approximately 10 minutes at 37° C. for virus M+, which has been firmly anchored to receptors in the cold, to release itself and that something less than 20 minutes in addition to this allows the development of almost complete dominance over a subsequently added virus. These time relations fit in well with the observations of Ishida and Ackermann (1956) who used a completely different ap-

proach. Their complex I is equivalent to the process of union to receptors in the cold, while the process by which, in their experiments, the system becomes insusceptible to washing with immune serum corresponds in time almost precisely with our development of dominance by the first added virus.

SUMMARY.

Interpolation of a period of chilling at 0° C. for 20 hours at any stage of the process makes very little difference in the sequence of influenza virus production at 37° C. in the de-embryonated egg.

Double infections with the strains M+ and WS— which interact to give doubly neutralized virus (phenotypic mixtures) and an easily isolated recombinant can provide information, based on the dominance of one strain over the other, about the early stages of infection which cannot be obtained from single infections.

When one virus is taken up by the cells in the cold before the other virus is added also in the cold, the virus presented *second* dominates in the yield. This dominance is annulled or reversed when the cells are pretreated with RDE.

When preparations on which one virus has been adsorbed in the cold are exposed to 37° C. before addition of the second virus, the first virus dominates the eventual yield if the intervening period is more than 10 to 30 minutes.

These indications of two initial stages in the process of infection are discussed in relation to the work of Ishida and Ackermann (1956).

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THE ACTION OF RIBONUCLEASE ON THE MULTIPLICATION OF INFLUENZA VIRUSES IN THE DE-EMBRYONATED EGG

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Under the influence of Brachet's studies on the influence of ribonuclease on protein synthetic actions in cells, Le Clerc (1956) examined the action of this enzyme on the process of influenza virus multiplication in allantoic cells. She found very great reduction in the amount of haemagglutinin produced both in the intact embryo and in fragments of chorio-allantois used according to the method of Tamm *et al.* (1953). In her opinion the effect of the ribonuclease was on ribonucleic acid (RNA) involved in the process of intracellular multiplication of the virus.

These observations seemed to be highly relevant to the work in progress in this laboratory on the nucleic acids of influenza virus (Ada and Perry, 1956) and on the genetic interactions between virus strains. In particular it seemed to offer a possible approach to the analysis of some of the stages of intracellular multiplication of influenza virus. We have therefore applied Le Clerc's method to the growth of virus in the de-embryonated egg using in most experiments our standard mixture of virus strains M+ and WS— so that any influence on recombination could be observed as well as the changes in overall haemagglutinin production.

First experiments indicated that the effect was rather slight but by successive modifications of technique an effect can now be demonstrated with relatively small amounts of the enzyme. The inhibitory effect persists for more than 24 hours after ribonuclease has been removed from the bathing fluid and it has not so far been proved practicable to use the technique for any analysis of the process of multiplication.

Materials and Methods.

Virus strains. Most of the work was done with our standard test viruses M+ and WS— which on recombination give the easily identifiable strain MEL. These have been fully described in earlier papers (Burnet and Lind, 1952; Lind and Burnet, 1953). Other strains

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used were the WSE and MEL strains of influenza A, RYAN, a filamentary A prime strain (see Burnet and Lind, 1957a), aWS–, an avirulent mutant of WS– (Lind, 1957) and the standard type B strain, LEE.

Diluents.

Ringer's solution. NaCl 0·85 gm., KCl 0·025 gm. and $CaCl_2$ 0·03 gm. made up to 100 ml. with distilled water. With the addition of 0·1 p.c. glucose, penicillin (40 units/ml.) and streptomycin (50 μg/ml.) this solution is referred to as GPSR.

Calcium-free Ringer's solution was also prepared by omitting the $CaCl_2$ component. With the addition of glucose and antibiotics this is referred to as GPSR–.

Normal saline. 0·9 gm. NaCl made up to 100 ml. with distilled water.

EDTA. The di-sodium salt of ethylendiamine-tetracetic acid.

Ribonuclease (RNase). This was prepared for us by Mr. H. F. Holden using Kunitz's method (1940) as modified by McDonald (1948). The preparation was essentially McDonald's "fraction B". A commercial specimen of protease-free enzyme (Worthington's Biochemicals Ltd.) used in two experiments gave the same level of effect.

De-embryonated egg (DEE) techniques. These have been described previously (Burnet and Lind, 1954a) and only minor modifications have been introduced. In outline, the eggs are de-embryonated after 14-16 days' incubation and the chorio-allantois well washed with Ringer's solution. Virus, normally a mixture of M+ and WS– in the form of equal parts of allantoic fluid virus previously diluted to 20 agglutinating doses (AD), i.e. to a haemagglutinin titre of 20, is left in contact at 37° C. for one hour and then the DEE thoroughly washed and refilled with GPSR. Harvests are normally made 7 hours and 22 hours after the virus is first added to the DEE.

In later experiments, DEE have frequently been held at 0-1° C. for some period in the sequence. For this purpose a rotor on which the eggs could be held at 15° to the horizontal, essentially similar to our standard 37° rotor, was installed in a cold room at 0-1° C. In changing from one temperature to another, the DEE were emptied and refilled with fluid at the new temperature before being placed on the appropriate rotor.

For most purposes a stock ribonuclease solution of 10 mg. per ml. was prepared and the appropriate amount added to the 5 ml. of fluid used in the DEE. In all experiments the amount of ribonuclease is expressed as the amount added per DEE, in most cases 3 mg., i.e. 0·3 ml. of the stock solution.

For most experiments only the haemagglutinin (HA) titres of the harvests were estimated. As a rule the fluids were titrated by dilution in (a) saline, (b) in anti-MEL 1:200, and (c) anti-WS serum 1:200 as described by Burnet and Lind (1954 a and b).

The sum of the titres obtained in the two antisera is expressed as a percentage of the titre in saline and the value obtained termed the "total percentage". Values of less than 50 p.c. were considered indicative of neutralization by both sera, *viz.*, double neutralization. Determination at the amount of double neutralization also allowed an estimate to be made of the amount of "phenotypic mixing" in the virus population of the harvests from the doubly infected DEE.

Preparation of extracts of chorio-allantoic membranes from DEE. The chorio-allantoic membranes were washed thoroughly with saline before removal from the DEE, drained, ground with alundum and suspended in 1 ml. of saline per membrane. The supernatants, after light centrifugation, were used for titration.

Complement fixation titration. The technique used was that described by Donnelley (1951) for detecting soluble complement fixing antigens. In the initial experiments the chorio-allantoic membrane extracts were freed of detectable haemagglutinin by successive adsorptions with human red blood rells; later this was considered unnecessary as the contribution of the haemagglutinin to the total CF titre was slight and could be accounted for by appropriate controls. The serum employed in the test was from a ferret previously in-

fected with an influenza A strain virus, and prior to the test this serum was adsorbed for some hours in de-embryonated eggs to remove any antibody against chorio-allantoic membrane. The titre is expressed as the reciprocal of the dilution of membrane extract showing 50 p.c. haemolysis.

EXPERIMENTAL.

The results of early experiments were disappointing. The addition of 3 mg. of RNase per DEE resulted in only a small reduction in HA production except with the poorly egg-adapted strain RYAN. Once the desirability of excluding Ca++ was recognised, however, the results were striking and reproducible. Only a few of the experiments carried out will be presented to establish the main points of the phenomenon.

Slow entry of RNase in the presence of Ringer's solution (GPSR). Ribonuclease, 3 mg. per DEE, was added at varying intervals before the addition of the M+/WS— virus mixture for 1 hour. Harvests made 22 hours after infection were titrated for their HA content with the results shown in Table 1.

TABLE 1.

Time required for RNase effect to develop.

Period between additions of RNase and virus	HA titres of 22 hour harvests	
Control (no RNase)	240	
½ hour	130.	70
1 hour	140.	120
2 hours	50.	40
4 hours	40.	30

Advantages of absence of Ca++. Pairs of DEE were given either 1 or 3 mg. of RNase 2 hours before the addition of virus. The solutions used in the four groups were Ringer (GPSR), Ca++ free Ringer (GPSR—), normal saline and normal saline + 1 p.c. EDTA. Throughout the manipulations each group received its own type of fluid, additions of the various reagents being made as required. Table 2 shows the haemagglutinin yields in duplicate after 22 hours.

TABLE 2.

Influence of Medium on Action of RNase.

RNase per DEE	HA titres of 22 hour harvests			
	Fluid medium			
	GPSR	GPSR—	Saline	Saline+1 p.c. EDTA
Nil	320, 150	350, 160	300, 360	240, 180
1 mg.	30, 50	24, 24	12, 40	8, 20
3 mg.	30, 7	<2, <2	<2, 4	<2, 4

There is no significant difference between the three media lacking Ca++, and Ca++-free Ringer (GPSR—) was adopted as the standard.

Activity of heat-treated RNase. To establish that the effects observed were due to RNase and not to protease contaminants, the RNase preparation was treated by Kleczowski's (1948) method. A solution containing 20 mg. of RNase per ml. was prepared in saline. Portion of this was diluted with an equal amount of M/5 borate buffer, pH 7·05, and heated at 100° C. in a boiling water bath for 10 minutes. The remaining portion was diluted with an equal amount of saline and left unheated.

Groups of 3 DEE were inoculated with 5 ml. of GPSR— containing 0·3 ml. and 0·1 ml. amounts of both the heated and unheated preparations (i.e. 3 mg. and 1 mg. of RNase) and, together with DEE containing GPSR— only, were incubated for 1 hour on the 37° C. rotor. All DEE were then inoculated with 0·5 ml. of a mixture of M+ and WS— diluted to give a final titre of 10AD of each virus in each DEE. After further incubation for 1 hour the DEE were emptied, thoroughly washed out and refilled with 5 ml. of GPSR—. Harvests were made 8 hours after infection, the eggs refilled and harvested again 13 hours later.

The results are shown in Table 3 and indicate that the activity of the heated RNase is not significantly less than that shown by the standard unheated preparation.

TABLE 3.

The effect on subsequent growth of virus of pretreatment of DEE with varying amounts of heated and unheated RNase.

Amounts of RNase added 1 hour before virus inoculation		HA titres of DEE harvests.					
		8 hours			22 hours		
Unheated RNase	3 mg.	<1	<1	<1	3	5	4
	1 mg.	1	1	1	12	12	10
Heated RNase	3 mg.	<1	<1	1	7	3	4
	1 mg.	1	4	2	10	16	16
Control (no RNase)		55	35	45	70	110	70

Influence of time relations of RNase and virus. In this experiment all DEE received the same inocula, 3 mg. RNase and 20AD of virus mixture, but the order of the additions and the time intervals between them were varied. In the first four sets RNase was given 2 hours before virus, 1 hour before virus, simultaneously with virus, and immediately after the virus had been removed. In each set virus was present for 1 hour and in the first three the RNase was discarded with the virus, the DEE then being recharged with GPSR—. RNase was left in the fourth set for 1 hour and then removed. The subsequent sets received RNase 1, 2, 3 and 4 hours after the first addition of virus (i.e. 0, 1, 2, 3 hours after its removal) and the enzyme was allowed to remain until the yield

was harvested. The virus content of chorio-allantoic membrane extracts was also estimated. The results are shown in Table 4.

TABLE 4.

Effect of time relations between virus and RNase.

Time of RNase addition in relation to virus		HA		
		22 hour harvest		Chorio-allantoic membrane
−2 hours	Removed	<2	<2	<2
−1 hour	Removed	<2	<2	<2
0	Removed	3	8	3·5
+1 hour	Removed	14	8	6
+1 hour	Retained	<2	18	1·5
+2 hours	Retained	12	2	1·5
+3 hours	Retained	20	30(A)	3
+4 hours	Retained	18	45(B)	3
No RNase		300	160(C)	128(D)

This experiment thus establishes that even 4 hours after infection has been initiated, RNase can produce a marked reduction in the amount of virus present in the fluid and even more strikingly in what can be extracted from the chorio-allantoic membrane (CAM).

In all the experiments that have been reported all fluids with HA titres above 10 were also tested for the extent of double neutralization. In this experiment the pools marked A B C D in Table 4 were tested with the results shown in Table 5.

TABLE 5.

Double neutralization of virus in preparations A, B, C and D (Table 4).

Fluid, &c.	HA titre in			Total Percentage[1]
	Saline	Anti M	Anti WS	
Pool A	30	0	7	30
B	30	<2	15	50
C	240	12	16	11
CAM[2] D	128	7	10	13

1. Defined under methods.
2. CAM = chorio-allantoic membrane extract.

It appeared from these results that, in the presence of RNase, less doubly neutralized virus was produced and that the MEL component tended to be dominant over WS. Several other experiments were therefore carried out to determine what effect partial inhibition by RNase had on the character of the yield from our standard M+/WS— infection.

Effect on RNase on double neutralization and relative yield of M and WS

haemagglutinin. Three sets each of eight DEE were used. In set A, virus M+/WS— alone was used. Seven hours after infection the fluids were harvested from all eight DEE and the chorio allantoic membranes from four. The remaining four DEE were refilled and the fluids and membranes harvested at 22 hours.

Set B received virus, as A, at zero time but after harvesting the fluids at seven hours, 3 mg. of RNase per DEE was added and left till both fluids and membranes were harvested at 22 hours.

In set C, RNase and virus were added together at zero time and both removed after 1 hour. Again harvests of fluids and membranes were made at 7 and 22 hours.

In Table 6 are shown the HA titres of both fluids and membrane extracts and the results of titrations against specific antisera. The feature of chief interest in Table 6 is the comparison of the 7 hour and 22 hour harvests from DEE in A 5, 6, 7, 8 which received no RNase with those of B 1, 2, 3, 4 which

TABLE 6.

Influence of RNase on the HA yield from DEE infected with a mixture of M+ and WS−.

Preparation	7 hour harvest			22 hour harvest		
	Saline	Anti M	Anti WS	Saline	Anti M	Anti WS
A 1	36	3·5	3			
2	30	3	1·5			
3	50	<2	28			
4	70	11	3			
5	60	11	3	90	30	8
6	120	3·5	9	250	15	25
7	40	2	5	150	10	30
8	120	3	10	300	10	40
B 1	40	4	1	12	3	<2
2	80	5	3	18	3	1
3	35	<2	15	6	<2	4
4	50	4	3	12	3	<2
C 1	<2	..	..			
2	<2	..	..			
3	<2	..	..			
4	<2	..	..			
5	<2					
6	<2			4	1	<2
7	<2			8	..	..
8	<2			8	3	..
CAM[1] A(1-4)	48	6	5			
(5-8)				70	8	12
CAM B				2	..	..
CAM C (1-4)	<1					
(6-8)				2		

A. No RNase. B. RNase added 7 hours after virus.
C. RNase and virus added together at zero time.
1. CAM = chorio-allantoic membrane extract.

received RNase immediately the 7 hour fluids had been collected. It will be seen that this late addition of enzyme greatly reduced the yield, but has relatively little effect on the pattern of relative dominance. In each instance this pattern is established at 7 hours and irrespective of whether or not RNase is added, it persists over the 7-22 hour period. Specimens A5 and B3 may be noted particularly.

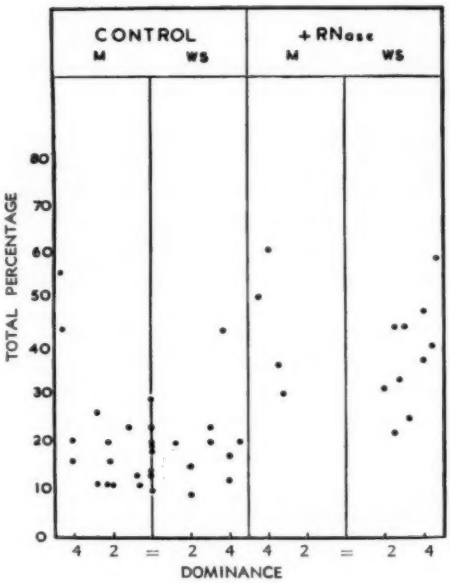

Fig. 1. Total Percentage: Sum of the HA titres obtained in antiM and antiWS serum diluents expressed as a percentage of the HA titre obtained in saline. Values of less than 50 p.c. are indicative of neutralization by both antisera.
Dominance: Dominance of M or WS serotypes in the yields from RNase treated and untreated DEE infected with M+/WS− mixtures.

Although the effect is only slightly evident in Table 6, the overall picture is consistent in showing less complete double neutralization when the yield is reduced by the action of RNase. Fig. 1 has been compiled from all suitable experiments to show that

(a) the great majority of yields from DEE inoculated with standard virus mixtures give "total percentage" values below 30 p.c. and a fairly symmetrical scatter of dominance of one virus over another;

(b) in both control and RNase groups there are a few points indicating almost selective overgrowth of M components without evidence of double neutralization;

(c) the RNase group shows much less evidence of double neutralization with an almost complete consistent bias toward dominance by the WS component.

Infectivity studies. Selected preparations from most experiments were titrated for infectivity by the allantoic route and sufficient limit positive fluids tested to establish the presence of the recombinants MEL or WSE in each. The results were remarkably uniform in showing that within experimental error the yield of infectivity was proportional to the HA titre. In line with previous experience the infectivity/HA ratio of fluids from DEE is found to be lower than that for standard fluids from intact embryos which in our convention give values close to $10^{5.6}$. Table 7 shows that all values obtained lie between $10^{4.3}$ and $10^{5.2}$. All fluids in Table 7 showed evidence of recombination usually by

TABLE 7.

Infectivity/HA ratios of selected preparations from DEE.

Type	Fluids	CAM extracts
Virus alone	4·8, 4·8	4·7
RNase 2 hours before virus	4·7	
RNase and virus together	5·0	5·0, 5·2
RNase 3-4 hours after virus	4·7	4·5±, 4·7

Infectivity/HA ratios shown in $\log_{10}$ units.

the isolation of one or more MEL fluids on limit dilution titration in chick embryos. No attempt was made to analyse the yield of recombinants in detail.

Recovery from action of RNase. In order to eliminate the possibility that the effect of RNase was simply a matter of functional destruction of most cell activities, it was necessary to show that the inhibitory effect could be eliminated, i.e. that after removal of RNase and appropriate washing the DEE again became capable of virus production.

In the experiment summarized in Table 8 six sets of DEE were prepared. The DEE in three of these each received 3 mg. of RNase in GPSR— and those in the other three GPSR— only. All were incubated for 2 hours on the 37° C. rotor, then emptied and washed out. One set of RNase treated and one of

TABLE 8.

Progressive loss of RNase activity in DEE maintained at 37° C. for varying periods between RNase treatment and inoculation with virus.

Period between beginning of RNase treatment and virus inoculation	Control no RNase				RNase 3 mg. 2 hours			
	Fluid		CAM[1]		Fluid		CAM[1]	
	6 hrs.	23 hrs.	23 hrs.		6 hrs.	23 hrs.	23 hrs.	
	HA	HA	HA	CFA[2]	HA	HA	HA	CFA[2]
2 hours	50 100 30	100 220 100	80	4	1·5 0·8 1·0	10 10 14	20	<2·5
24 hours	35 50 30	140 120 200	60	7·5	14 12 8	32 18 14	50	2·5
48 hours	15 20 6	64 140 60	120	10	16 20 16	70 120 70	150	7

1. CAM = extract of pooled chorio-allantoic membranes.
2. CFA = titre of soluble complement fixing antigen.

untreated DEE immediately received the standard mixture of M+ and WS—. The remaining sets were filled with GPSR— and maintained on the 37° C. rotor with twice daily changes of the bathing fluid. At 24 and at 48 hours after the beginning of the experiment an RNase-treated and an untreated set of DEE from this latter group were inoculated with the virus mixture. In all cases the DEE were washed out one hour after virus inoculation and refilled with GPSR—. Harvests were made 6-7 hours and again at 22-24 hours from the time of adding the virus. The membranes of some of the DEE inoculated at 48 hours became partially detached from the shell membrane before the conclusion of the experiment, but the yields from these showed no divergence from those with intact membranes and all values are included in Table 8. Titres of soluble complement fixing antigen (CFA) in the membrane extracts were also determined. Table 8 shows that 48 hours after treatment with RNase the DEE have become equivalent in all respects to DEE which have received no enzyme.

Experiments using a period of chilling. In another paper it is shown that a period of 16-20 hours' chilling can be interpolated anywhere in the sequence of virus multiplication in the DEE with virtually no modification in the yield (Burnet and Lind, 1957b).

This has allowed a considerable extension of the work with RNase (a) in devising a method by which the activity of much smaller amounts of RNase can be demonstrated, and (b) in showing that the RNase effect is not lost at 0° C. in the way it is when RNase-treated DEE are maintained at 37° C.

Experiments indicated that the most effective method of demonstrating RNase action was to allow the enzyme to act for two hours at 37° C. and then to leave the DEE, after washing, at 0° C. for 16-20 hours. The virus was then added and the DEE left for two hours in the cold. After washing again the DEE were placed on the 37° C. rotor and harvested after 20-22 hours' incubation. Table 9 shows the results obtained using 0·3 mg. of RNase per DEE.

As in all such experiments there is marked variation in the results obtained from individual DEE, particularly in the set receiving virus at 37° C., but the effect of 0·3 mg. RNase per DEE is quite significant and is most noticeable when two hours' exposure to RNase at 37° C. is followed by 16-20 hours at 0° C. before virus is added also in the cold. This sequence has been used in most of the later experiments and is referred to as the "optimal cold method".

In a larger series of DEE in which the effect of 1 mg. of RNase per DEE was tested by the "optimal cold method", special note was made of DEE in which the chorio-allantoic membrane had become detached from the shell membrane. In this series extracts of chorio-allantoic membranes were titrated individually for their content of HA and of soluble CFA. The DEE were inoculated with 1 mg. of RNase and incubated for two hours at 37° C. They were then washed and left overnight at 0° C. and the next morning inoculated with the standard M+/WS— virus mixture. After one hour's incubation at 37° C. the

TABLE 9.

Inhibition of HA production by RNase using different techniques.

Sequence of treatment of DEE		No. of DEE	Character of 22 hour harvest		
			HA[1]	Relative dominance[2]	Total[3] Percentage
	Virus 1 hour 37° C.	5	52±15	2·3	33
0·3 mgm. RNase 2 hours 37° C.	Virus 2 hours 0° C., 1 hour 37° C.	5	37±4	6·1	31
	GPSR—2 hours 0° C., virus 2 hours 0° C.	5	31±5	5·1	36
	GPSR—20 hours 0° C., virus 2 hours 0° C.	5	22±5	5·0	20
No RNase	GPSR—20 hours 0° C., virus 2 hours 0° C.	5	140±12	2·8	11

1. The mean HA titre is shown ± the Probable Error.

2. HA titres determined in saline in anti-M serum/200 and anti-WS serum/200. To determine Relative Dominance (R.D.) of M or WS serotype in the yield, the titres obtained in anti-M and anti-WS are each converted to a percentage of the saline titre and expressed as a fraction.

$$\frac{\text{p.c. titre in anti-M}}{\text{p.c. titre in anti-WS}} = \text{RD}$$

3. The sum of the HA titre obtained in anti-M and anti-WS expressed as a percentage of the saline titre.

DEE were washed again, refilled with GPSR— and harvests were made after 7 and 22 hours' incubation at 37° C. An equal number of DEE which had not been treated with RNase was included in the series. The HA titres of the harvested fluids and the HA and CFA titres of the membrane extracts are shown in Table 10.

TABLE 10.

Effect of 1 mg. of RNase per DEE tested by the "optimal cold method".

Preparation	Titre Determined	Average titres[1]			
		RNase-treated DEE		Untreated DEE	
		7 hr.	22 hr.	7 hr.	22 hr.
Fluid	HA (all DEE)	<1	7·7±1·4	8·0±1·1	83±9
	HA (excluding fluids from DEE with torn or detached membranes)	<1	5·1±1·0	8·4±1·2	64±2
CAM[2]	HA		11·9±2·1		102±13
	CFA		11±1·7		20±1·5

1. The titres shown are the average of harvests from 10 DEE ± Probable Error.
2. CAM = extracts of chorio-allantoic membranes.

There is a considerable scatter of values and some improvement is seen if membranes which might have allowed chorionic multiplication of virus are excluded.

The inhibitory effect of RNase is not lost if DEE, which have been treated with RNase and then washed out, are held at 0° C. for up to 96 hours before the inoculation of virus. This was shown both with 3 mg. and 1 mg. of RNase per DEE. In the latter experiment groups of 3 DEE were used. The experimental lots were treated with 1 mg. of RNase for two hours at 37° C., while the controls received only GPSR— for this period. All were then chilled and placed on the cold rotor for the periods shown in Table 11 before receiving virus for two hours at 0° C. After inoculation with virus the DEE were washed out, refilled with 5 ml. of GPSR— and incubation continued at 37° C. Harvests were taken at 7 and 22 hours and titrated for HA; chorio-allantoic membranes were removed at 22 hours and extracts prepared from them were titrated for HA and soluble CFA. The results, shown in Table 11, are in sharp contrast to those in Table 8, which show the progressive disappearance of the RNase effect when the DEE are maintained at 37° C.

TABLE 11.

Maintenance of RNase effect in the cold.

Time on cold rotor	RNase 1 mg.				No RNase			
	Fluid		CAM[1] 22 hr.		Fluid		CAM 22 hr.	
	7 hr. HA	22 hr. HA	HA	CFA	7 hr. HA	22 hr. HA	HA	CFA
24 hours	2	24	11	2·5	37	80	140	27
48 hours	2	5	6	<2·5	20	90	240	33
72 hours	2	13	1·5	2·5	1	60	60	13
96 hours	<1	4	2	2·5	5	27	60	7

1. CAM = chorio-allantoic membrane extract.

Comparative action of RNase on different strains of influenza virus. There appear to be only minor differences between influenza virus strains in regard to their susceptibility to inhibition by RNase. In experiments carried out at 37° C., suitable dilutions of strains WSE, aWS—, RYAN, MEL and LEE were inoculated into groups of DEE each previously treated for two hours with 3 mg. RNase. Harvests taken at 22 hours from DEE inoculated with WSE, aWS— and RYAN strains all gave HA titres of < 1, while those inoculated with MEL and LEE gave titres 2-5 p.c. of those given by their respective controls.

Using the "optimal cold method", a comparison was made of the four virus strains used in our genetic work, MEL, M+, WS— and WSE. The results are shown in Table 12.

TABLE 12.

Comparative action of RNase on virus strains MEL and WSE and their derivatives.

Dose of RNase	MEL				M+			
	Fluid HA		CAM 22 hr.		Fluid HA		CAM 22 hr.	
	7 hr.	22 hr.	HA	CFA	7 hr.	22 hr.	HA	CFA
1·0 mg.	5	35	15	20	<1	5	<1	10
0·1 mg.	7	130	60	40	9	40	5	10
Nil	10	170	100	46	17	90	12	15
	WS−				WSE			
1·0 mg.	<1	8	5	8	<1	1	<1	5
0·1 mg.	4	28	15	8	11	37	1	10
Nil	2	40	60	20	37	107	6	27

The differences are not striking, the order of increasing inhibition being MEL, WS—, M+, WSE. It may be of interest to note that this is the order of frequency with which recombinants are obtained in experiments with this system.

Soluble complement fixing antigen in membranes. In all the later experiments, the chorio-allantoic membranes were removed at the final harvest and extracts prepared for titration of HA and soluble CFA. Typical results are included in Tables 8, 10, 11 and 12.

In general, the use of RNase reduced both titres but the HA content was always proportionately more affected. Averaging the whole series of results, not all of which were strictly equivalent, the HA content of membranes from DEE treated with 3 mg. or 1 mg. of RNase was 10 p.c. of that from control membranes, whereas the soluble CFA content averaged 40 p.c. of the control.

DISCUSSION.

These results confirm those reported by Le Clerc (1956). In all probability the effect of RNase is on some aspect of the intracellular process. We can confirm that there is no evidence of action on free virus or of any process blocking the adsorption of virus to the cell surface. The importance of RNA in influenza virus multiplication is well established and the activity of RNase must indicate that some phase of multiplication involves RNA (not necessarily of viral type) in a form accessible to the action of the enzyme. It is clear from the marked effect of RNase added several hours after infection had been initiated that the susceptible phase is one that persists throughout the period of virus multiplication and release, but no information has been gained to allow any

closer analysis of the process. The fact that with almost complete regularity the production of virus is diminished to a much greater extent than the production of soluble complement fixing antigen may become of importance when more is understood of the relation of the soluble antigen to the process of multiplication.

Our original hope that by the use of RNase in experiments on recombination, some analysis of the recombination process in the cell would be possible, has not been fulfilled. The insuperable difficulty has been the prolonged effect of the enzyme once it has been introduced into the susceptible cells.

SUMMARY.

Ribonuclease is capable of inhibiting the production of haemagglutinin in de-embryonated eggs infected with influenza viruses.

The inhibitory effect is increased (a) if time is allowed for penetration of ribonuclease before the virus is added, (b) if Ca^{++} is eliminated from the saline medium used, and (c) if the DEE are chilled before adding virus.

Ribonuclease greatly reduces the subsequent yield of haemagglutinin both in the fluid and in the chorio-allantoic membrane if added after virus production is in full progress.

When a suitable mixed inoculum is used, the degree of double neutralization (phenotypic mixing) shown by the harvested virus is reduced if ribonuclease has been used. No evidence of any increase in incomplete virus was obtained.

The inhibitory effect of ribonuclease persists for at least 96 hours if the de-embryonated egg is held at 0° C. but is lost in 48 hours at 37° C.

There are only minor differences in the susceptibility of different virus strains to inhibition by ribonuclease.

The content of soluble complement fixing antigen in the chorio-allantoic membrane is reduced, but to a much smaller extent than the haemagglutinin content.

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FURTHER STUDIES OF RECOMBINATION BETWEEN HEAT-INACTIVATED VIRUS AND ACTIVE VIRUS

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Probably the most convincing proof of the existence of recombination between influenza viruses is to show that genetic components of the progeny can be contributed by non-viable virus used as one 'parent' in the system. This was first demonstrated by Burnet and Lind (1954a) using heat-inactivated virus and has since been confirmed by Baron and Jensen (1955) and Hirst and Gotlieb (1956) using mainly virus inactivated by ultra-violet light. All workers have used the principle of isolating from the primary product of double infection clones of the serological type of the inactive parent by subinoculation with antiserum against the active parent.

Both the more recent groups of workers showed that a proportion of recombinants can be obtained even if the inactivated virus were inoculated 2 or 3 days before the active component. This was of particular interest to us in view of our interest in the possible use of ribonuclease treatment for the analysis of the process taking place in doubly infected cells. If cellular infection by the two parent viruses could be separated by a period of 24 hours or more, it might be possible to use ribonuclease or other reagents in the intervening period and to get rid of them before adding the second component. For this reason, a re-examination of the behaviour of heat-inactivated virus in recombination experiments was undertaken using our standard pair of viruses M+ and WS— from which, by recombination, MEL and WSE types can be produced.

Although it was not possible to develop a system suitable for systematic study with ribonuclease, a number of interesting findings were encountered, in particular the asymmetric relation of the two parent types in regard to recombination and interference. In general, heat killed M+ readily produced recombinants but did not provoke interference with the growth of subsequently added WS—. Heat-inactivated WS—, on the other hand, gave a very poor yield of recombinants but was active in interfering with the growth of M+ subsequently added.

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MATERIALS AND METHODS.

Virus strains. The strains M+ and WS– have been used in much work of this type in recent years (Lind and Burnet, 1953; Burnet and Lind, 1954a). Their phenotypic character can be represented M+ (ABDF – ce), WS– (abdf – CE) where the symbols represent characters fully described previously (Lind and Burnet, 1954). In brief, Aa concerns serological type as determined by haemagglutinin inhibition reactions; Bb, heat stability of haemagglutinin; Cc and Dd, development on simple heating of indicator state against semi-purified mucoids from meconium and from sheep salivary glands respectively; Ee, capacity to produce haemorrhagic lesions in the chick embryo after inoculation on the chorio-allantois; Ff, virulence on intranasal inoculation into mice.

The recombinants MEL (ABDF-CE) and WSE (abdf-ce) were identified in the first instance by *in vitro* tests for the characters Aa, Cc and Dd. Where necessary the other characters were tested for by the appropriate means.

Recombination technique. No significant alteration has been made in the methods of using de-embryonated eggs DEE, except where a period of 18 hours or more elapsed between the two inocula (Burnet and Lind, 1954b). For such experiments the first inoculation of inactivated virus was made into the allantoic cavity of the intact embryo. De-embryonation was carried out just before the addition of the living virus component.

Eggs were used on the 14th-16th day of incubation. The shell was removed around the end away from the air sac, the eggs de-embryonated, washed with Ringer's solution and inoculated with virus diluted usually to 10 agglutinating doses in a volume of 5 mls. of Ringer's solution containing 0·1 p.c. glucose, penicillin 40 units per ml and streptomycin 50 μg. per ml. (GPSR). The inoculum was removed and the preparation washed with warm Ringer's solution after one hour on the turntable at 37° C. The DEE were then refilled with warm GPSR. Harvests were made at 7 hours and, after refilling and leaving overnight, at 22-23 hours. The 7-hour harvest was titrated for haemagglutinin and for the presence of viable virus of serological type corresponding to the inactive parent by inoculation of 11-day embryos with dilutions made in 1:20 immune serum prepared against the active parent. Thus when heat-inactivated M+ was used the primary fluid containing a great excess of WS– was diluted serially so that each virus dilution also contained anti-WS immune rabbit serum at a concentration of 5 p.c. Under these conditions the titre of WS– fluids is reduced by 6 $\log_{10}$, so that only after inoculation of undiluted primary fluids are any WS– fluids to be expected in the yield. All positive fluids obtained by inoculation of the two highest infective dilutions in these titrations were characterized for Aa, Cc and Dd, which allowed recognition of MEL AD-C or WSE ad-c recombinants. Except for special reasons, no further characterization of these fluids was undertaken.

EXPERIMENTAL.

Inactivation of virus fluids. Freshly harvested allantoic fluid virus from embryos inoculated at 11 days and incubated for 44 hours was diluted with half volume of M/5 borate buffer (0·19 M boric acid, 0·0025 M sodium tetraborate) at pH 7·0. It was then distributed in 5 ml. ampoules and heated, completely immersed, in an accurately controlled water bath for the time stated. For inactivation at 37° C. the fluids were used undiluted and the ampoules immersed in a jar of water kept in a bacteriological incubator at 37° C. The time for inactivation at this temperature was 14-21 days, samples being taken twice weekly and the ampoules being removed as soon as a negative infectivity test was obtained. In all infectivity tests, fluids from eggs negative 3 days after

TABLE 1.

Result of heating M+ and WS— at different temperatures with fluids buffered with M/15 borate pH 7·0.

M+				WS—			
Temperature	Time	HA	Infectivity	Temperature	Time	HA	Infectivity
Unheated		330	10^8	Unheated		850	$10^{8.5}$
55° C.	30 min.	120	+	55° C.	30 min.	500	+
	60 min.	100	+		60 min.	320	+
	120 min.	90	+		120 min.	110	0/6 10^0
	180 min.	80	0/6 10^0		180 min.	30	0/6 10^0
57·5° C.	30 min.	100	+	57·5°C.	30 min.	25	0/6 10^0
	60 min.	80	0/6 10^0		60 min.	4	0/6 10^0
	120 min.	80	0/6 10^0		120 min.	<1	0/6 10^0
60° C.	15 min.	60	0/6 10^0	60° C.	15 min.	<1	—
	30 min.	40	0/6 10^0		30 min.	<1	—
	60 min.	25	0/6 10^0		60 min.	<1	—

inoculation were subinoculated and shown to contain no virus. The results at temperatures of 55°, 57·5° and 60° C. are shown in Table 1. Only those preparations were used which were free of viable virus as shown (a) by 6/6 negative eggs from 0·05 inoculum of undiluted virus with negative result on subinoculation of 1 ml. volumes from each egg, and (b) by failure to produce any haemagglutinin or cultivable virus when tested at 10AD level in DEE. The WS— fluids heated at 60° C., in which all haemagglutinin had been destroyed, were not tested. Those available therefore were

M+ 55° C. 180 min., 57·5° C. 60 min. 120 min., 60° C. 15 min. 30 min. 60 min.

WS— 55° C. 120 min. 180 min., 57·5° C. 30 min. 60 min. 120 min.

Each of these was tested (i) when administered to embryos undiluted in 0·25 ml. dose 18 hours before de-embryonation and addition of living complementary virus, and (ii) mixed at 10AD level with 10ADs of the living complementary virus and inoculated into DEE. In Table 2 are shown the results in terms of the yield of MEL from inactive M+ and of WSE from inactive WS—.

It will be seen that the best yield of recombinants from heated M+ is obtained when the two components are inoculated together and that at each temperature positive results are obtained only with the fluid heated for the minimal time needed to destroy infectivity completely.

Incidentally to these recombination experiments the haemagglutinin titres of the primary fluids were recorded as indices of interference by the heated virus given 18 hours previously. There was no significant reduction of WS— titres by any form of heated M+ given on the previous day. Despite minor irregularities the results in the opposite direction with heated WS— and active M+ showed highly significant interference. In Table 3 the titres obtained are shown in

TABLE 2.

Recombination of heated virus with complementary active virus.

Heated virus			Active virus	Method of testing	Yield of recombinants on titration of primary fluid		
					10^0	10^{-1}	10^{-2}
M+	55° C.	180 min.	WS−	18 hours*	3/4	0/4	0/4
				Together	4/4	4/4	3/4
	57·5° C.	60 min.		18 hours	1/4	0/4	0/4
				Together	4/4	2/4	0/4
	57·5° C.	120 min.		18 hours	0/4	0/4	0/4
				Together	0/4	0/4	0/4
	60° C.	15 min.		18 hours	1/4	0/4	0/4
				Together	1/4	0/4	0/4
WS−	55° C.	120 min.	M+	18 hours	0/4	0/4	0/4
				Together	0/4	0/4	0/4
		180 min.		18 hours	0/4	0/4	0/4
				Together	0/4	0/4	0/4
	57·5° C.	30 min.		18 hours	0/4	0/4	0/4
				Together	0/4	0/4	0/4
		60 min.		18 hours	0/4	0/4	0/4
				Together	0/4	0/4	0/4

M+ 60° C. 30 and 60 minutes and WS− 57·5° C. 120 minutes were similarly tested with completely negative results.

* 18 hours. Inactive virus inoculated into allantoic cavity 18 hours before de-embryonation.

terms of the percentage of the mean haemagglutinin titre (HA) of the control M+. The interfering effect of WS— virus inactivated at 37° C. was particularly marked and the result shown was repeated on several occasions.

A heat labile strain of M+ and its behaviour on recombination. In the course of this work an interesting anomalous strain was encountered in experiments with a batch of M+ virus heated to 56·5° C. for 30 minutes. This had retained minimal infectivity giving 3/8 positive results when inoculated un-

TABLE 3.

Interference by heated WS− with growth of M+.

Pretreatment of WS− given 18 hours before M+		HA titre of M+ in harvest (p.c. of control value)	
37° C.	18 days	<1,	<1
55° C.	120 min.	13,	33.
	180 min.	67,	81.
57·5° C.	60 min.	10,	30.
	120 min.	55,	108.
60° C.	15 min.	70,	33.
	30 min.	110,	48.

diluted and 1/8 at 10^{-1}; its HA titre was 50 as against the original fluid's HA titre of 600.

The usual type of recombination experiment was carried out using both preliminary inoculation of the heated M+ 18 hours before de-embryonation and simultaneous addition of the two viruses. Analysis of the first cycle yield in anti-WS serum gave the results shown in Table 4.

TABLE 4.

Recombination experiment from which heat-labile variant of M+ was obtained.

Inoculum before de-embryonation	Inoculum after de-embryonation	Yield in anti-WS serum
Nil	Heated M+	Nil
Nil	Heated M+/active WS−	10^{-1} 3 MEL 10^{0} 4 MEL
Heated M+	Nil	Nil
(1) Heated M+	Active WS−	10^{-1} 1 MEL, 3 M+(L)
(2) Heated M+	Active WS−	10^{-1} 6 M+(L) 10^{0} 4 M+, 1 M+(L), 1 MEL(L)

M+(L) and MEL(L) fluids differing from standard M+ and MEL in having thermolabile haemagglutinin completely inactivated by heating for 30 minutes at 56° C.

In two experiments the heated M+ gave rise, in the presence of active WS—, to heat labile M+ clones, M+(L), and one similarly labile MEL strain. These were only obtained when the partially inactivated material was given before de-embryonation and only in the presence of active WS—. Four strains of M+(L) were fully characterized. All showed complete destruction of haemagglutinin on heating to 56° C. for 30 minutes and only a slight loss of titre at 50° C. Heating at the latter temperature converted M+(L) to the indicator state with the typical reactions for M+, viz. inhibition by meconium inhibitor but not by sheep salivary mucin. The virulence of all four strains for mice and chick embryos was of the level characteristic for M+ strains.

Recombination experiments with M+(L) and WS— gave a high yield of recombinants, the dominant form in the first cycle fluid having MEL characters except for heat lability of haemagglutinin at 56° C. A detailed titration of the first cycle fluid gave the results in Table 5.

TABLE 5.

Recombination between M+(L) and WS−.

Dilution of primary fluid	Eggs positive	Strains present
$10^{7.2}$	2/20	MEL(L)(2)
$10^{6.9}$	4/25	MEL(L), WS−, M+(L), DN*
$10^{6.6}$	6/20	1 WSE (others M serology)

*Fluid showing double neutralization from which M+(L), MEL(L) and WS− were isolated.

TABLE 6.

Comparison of labile types with standard M+ and MEL.

Fluid	Serology	HA		Inhibition of HA of virus heated 50° C. 30[1]		Pathogenicity	
		Unheated	56° C.	Mec.[1]	Sh. M[2]	Mice	CA
M+(L) 1	M	280	<1	+	–	0·2	6·0
M+(L) 2	M	640	<1	+	–	0·4	3·3
MEL(L) 1	M	800	<1	–	–	0·45	0·0
MEL(L) 2	M	500	<1	–	–	0·1	0·0
Stock M+	M	330	120	+	–	0·5	5·5
Stock MEL	M	800	800	–	–	0·6	0·0

1. Meconium.
2. Sheep Salivary Mucin.

Mice: Extent of lung lesions according to standard convention.
CA: Extent of haemorrhagic lesions in embryo. (See Lind and Burnet, 1954.)

The characters of 2 MEL(L) recombinants and of 2 M+(L) strains are shown in Table 6.

The appearance of a heat labile M+ strain raised a number of points of interest in regard to the mechanism by which it arose, but as no other preparation of heated M+ gave rise to this type in several further experiments, no experimental basis for discussion exists.

The point of chief interest is that the labile strain retains all other characters of M+ including the capacity to produce a high yield of recombinants with WS—. The MEL recombinants obtained are heat labile; the one WSE recombinant tested had all the normal characteristics of its type. The results are perfectly regular corresponding to the scheme.

$$\text{AbDF} - \text{ce} \times \text{abdf} - \text{CE} \rightarrow \text{AbDF} - \text{CE and abdf-ce}$$

and provide another example of the consistent association of the marker characters in the first "linkage group".

Behaviour of virus inactivated at 37° *C.* If allantoic fluid virus is held in sealed ampoules at 37° C. there is a gradual fall in infectivity with little loss of haemagglutinin titre. In our experience, inactivation is complete in 14 to 21 days. The experiments to be reported were all carried out with a single batch each of M+ and WS— fluids which gave negative tests for infectivity on the 14th day and were removed to a dry ice refrigerator three days later.

Each fluid was tested for infectivity undiluted and diluted 1:10, with subinoculation of the negative fluids to a further series of embryos. These tests showed no evidence of infectivity and the control tests made with each recombination experiment were equally negative. The characteristics of two such fluids are shown in Table 7.

TABLE 7.

Characteristics of fluids inactivated at 37° C. for 17 days.

Fluid	Original	After treatment at 37° C.
M+ 79241 WS− 79253	HA 600 ID_{50} $10^{-8.0}$ HA 1280 ID_{50} $10^{-8.2}$	HA 600 Noninfective HA 650 Noninfective

Experiments along the same lines as those with viruses inactivated at higher temperatures gave the results shown in Table 8.

TABLE 8.

Recombination with virus inactivated at 37° C.

Heated virus	Active virus	Method of testing*	Primary harvest			
			HA	Recombinants isolated		
				10^{0}	10^{-1}	10^{-2}
M+(37)	WS−	18 hours	200 160	2/6[1.]	1/6[1.]	0/6
M+(37)	WS−	1 hour	160 200	5/6[1.]	1/6[1.]	0/6
M+(37)	Nil	—	0, 0	0/6		
Nil	WS−	—	240			
			400			
WS−(37)	M+	18 hours	<1 2	0/6	0/6	
WS−(37)	M+	1 hour	380 350	0/6	0/6	
WS−(37)	Nil	—	0, 0	0/6		
Nil	M+	—	150			
			500			

* 18 hours—heated virus inoculated 18 hours before de-embryonation.

1 hour—heated virus added after de-embryonation and left for one hour before active virus added.

[1.] These recombinants were all of MEL character.

A considerable number of experiments were made with these materials in the hope of finding a method by which a much larger yield of recombinants could be obtained. In particular an attempt was made to assess the effect of

varying the amount of inactive virus given 18 hours before de-embryonation. The result obtained was quite definite, but very difficult to interpret. No significant relation was evident between dose and yield of recombinants in the system M+(37° C.)/active WS— as will be seen from Table 9. Similar experi-

TABLE 9.

Yield of MEL recombinants in relation to dose of inactive M+(37°) C. virus given 18 hours before de-embryonation and addition of a constant dose of active WS— virus.

Dose of M+ inactive virus	Yield of MEL recombinants per 0·5 ml. of primary fluid
Undiluted	100, 5, 2, 2, 0·5, 0·5
1:2	10
1:4	2·5
1:10	4, 4
1:20	10
1:32	0
1:100	5

Results from several experiments are pooled.

ments with WS— as the inactive component gave an extremely small yield of recombinants, three WSE strains being obtained from 12 primary fluids titrated. Interference with the growth of M+ was well marked in all experiments where undiluted WS—(37° C.) was used but irregular with diluted material. For obvious reasons it is much more convenient to examine the primary fluids from these recombination experiments for recombinants of the same serological character as the inactive "parent". With the inactive M+/active WS— system it is possible to provide a selective environment for the isolation of the second recombinant WSE by inoculating on the chorio-allantois and isolating virus from the embryo lung. In this way three strains of WSE have been obtained and characterized. There is no equivalent means of obtaining MEL recombinants from the inactive WS—/active M+ primary fluids.

DISCUSSION.

The objective of these experiments was to obtain a system in which a relatively prolonged period could be provided between the introduction of the two parent strains without diminishing the yield of recombinants. There is no doubt that inactive virus produced either with heat or by exposure to ultraviolet light can provide genetic units to react with living virus introduced up to three days later (Baron and Jensen, 1955), but the yield of recombinants is minute compared with what can be obtained when the same viruses are used simultaneously and in the active state. The yield was, in fact, too small for it to be worth while testing the influence of ribonuclease on the system.

The most interesting finding was the asymmetry of the results obtained with virus inactivated at 37° C. With M+ as the killed component there was very

little interference with the growth of WS— and a regular, if not profuse, production of MEL recombinants. With WS— as the inactivated component interference was marked but there was only very rare production of WSE recombinants. The same contrast was almost equally evident in the experiments using virus inactivated at higher temperatures.

No satisfactory explanation is available for the virtual absence of influence of dosage on the yield of recombinants, but the possible contributory factors are so numerous that only much more extensive experimentation would justify discussion.

The results indicate, as would be expected, that destruction of the capacity to provide genetic components follows very close on the heels of destruction of infectivity at all the temperatures used in the present work. It should perhaps be stressed that all the inactivated preparations used showed no evidence of multiplicity reactivation when inoculated in large doses and no evidence was obtained of reactivation or recombination when both M+ and WS— were used together in the inactivated state.

For the time being the fact that virus inactivated by any conventional criterion can contribute genetic components to progeny in the presence of a suitable second virus, is merely something to be kept in mind in any eventual interpretation of the process of influenza virus multiplication. The suggestion that interfering power may be inversely correlated with capacity to contribute to recombination may also become important but needs to be substantiated by a wider experience before any stress can be laid on it.

SUMMARY.

A study has been made of the capacity of heat-inactivated influenza virus to contribute to recombination reactions with an appropriate active virus as the second component.

Only virus which was non-infective by rigid criteria was used as the inactive partner.

The following temperatures, 37°, 55°, 57·5° and 60° C. were used, the exposures being arranged so that the minimal time necessary for complete inactivation was included.

With M+ as the heat-inactivated component, MEL recombinants were obtained in each case with the material having received minimal exposure. None of the M+ material given 18-20 hours before adding WS—(active) showed any significant interference. Using M+ inactivated at 37° C. the yield of recombinants was almost unrelated to the size of the dose of inactivated virus inoculated 18-20 hours previously.

With WS— as the inactivated component and M+ as the active one, there was very little production of WSE recombinants, a total of three strains being obtained, all from systems containing virus inactivated at 37° C.

However, WS—, inactivated at 37° C. and administered 18 hours before M+, strongly interfered with growth of the M+.

In a single experiment in which M+ which had not been completely inactivated was used with active WS—, an unusual heat labile variant of M+ was obtained.

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THE EFFECT OF BUTAZOLIDINE ON THE ANAPHYLACTIC LUNG

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It has been shown previously that sodium salicylate, *para*-aminosalicylic acid, sodium *para*-aminosalicylate, acetyl salicylic acid, aminophylline and colchicum inhibit the release of histamine and S.R.S. from the perfused anaphylactic lung of the guinea pig in a concentration corresponding to the effective therapeutic level employed in clinical conditions regarded by many as having an allergic basis (Trethewie, 1951a, b, 1952, 1954a, 1955a). It has been reported (Trethewie, 1955b, 1956; Mongar and Schild, 1956; Lecomte and Hughes, 1957) that butazolidine (B.T.Z.) inhibits the release of histamine in anaphylaxis. In this paper a detailed analysis of quantitative studies including evidence of inhibition of release of S.R.S. as well as of histamine by B.T.Z. is presented and certain clinical implications are discussed.

Method.

Guinea pigs were sensitized by the intravenous injection of 15 mg. crystalline egg albumin dissolved in sterile saline (0·5 ml.). The animals weighed 80-100 gm. and perfusion experiments were carried out 5-20 weeks later, when they weighed 300-500 gm. For these perfusion experiments the animals were stunned by a light blow on the head, the lungs were perfused through the cannulated pulmonary artery with Tyrode, excised and set up in an incubator at 37° C. The venous and lymphatic effluent was collected over a paraffin block while the lungs were ventilated under positive pressure through the trachea at 11 strokes per min. As the perfusion proceeded the ventilating pressure was gradually increased, but not so much as to force air into the pulmonary vessels. This manoeuvre was carried out so as to remove most of the oedema fluid. Perfusate so collected was boiled, immediately cooled, and tested on the isolated jejunum of the guinea pig. The substance producing immediate contraction of the gut under these circumstances has been shown to be histamine (Bartosch, Feldberg and Nagel, 1932) and further confirmed with antihistamine studies (Trethewie, 1951b). Histamine was estimated as histamine acid phosphate, and specific inhibition by anti-histamine confirmed the histamine assay. Slow reacting substance released in anaphylaxis (S.R.S.) (Kellaway and Trethewie, 1940), was likewise assayed.

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B.T.Z. was dissolved in an acid ether mixture, filtered, precipitated by evaporation, and neutralized with alkali. Portions of the dried B.T.Z. so obtained were weighed and taken up in Tyrode, to which after a few minutes mixing, 0·15 ml. of 2·993 N NaOH solution was added per 100 mg. B.T.Z. to bring the B.T.Z. fully into solution. The appropriate volume of solution was then poured into a Tyrode delivery flask, warmed to 37° C., and the lung perfusion continued with B.T.Z. in a concentration of 0·625-10 mg./100 ml. After collecting perfusate for 4-10 min., flowing at a rate of 1-2 ml./min., 20 mg. crystalline egg albumin dissolved in 0·5 ml. Tyrode was injected into the cannulated pulmonary artery immediately after adding B.T.Z. to make the concentration of B.T.Z. equal to that in the perfusing fluid. The flow was interrupted for one and a half minutes and then re-established. Perfusate was then collected 0-5 and 5-15 minutes later. After perfusion the lung was squeezed tightly between filter papers, ground up with silica, extracted with Tyrode and the resultant mixture was boiled.

EXPERIMENTAL.

Control Experiments. After the sensitizing procedure described above, all guinea pig lungs tested have been found to release histamine following the intra-arterial injection of crystalline egg albumin. The outputs of histamine in two such control experiments in this series were 0·3 μg. and 1·6 μg. in the first five minutes following injection, and 0·6 μg. and 0·4 μg. in the succeeding ten minutes. These findings are shown in Fig. 1, where the output is estimated

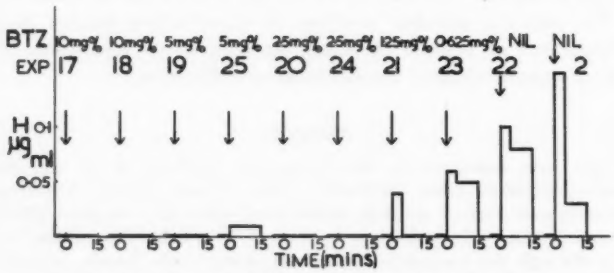

Fig. 1. Output of histamine from the isolated perfused lung following the injection of antigen in the presence of varying concentrations of Butazolidine.
Abcissa: time in mins. following injection (at the arrow).
Ordinate: output of histamine in μg./ml.

as histamine concentration in μg. per ml. (Exp. 2 and 22). The lungs in these instances contained 4 μg. and 9 μg. histamine per gm. respectively when squeeze-dried between filter papers after perfusion. Similarly, there was a considerable output of S.R.S. in each instance. This finding is illustrated in Fig. 2 where the responses to boiled perfusate from Exp. 22 are recorded. It is noted that following the injection into the bath of 1 ml. perfusate obtained 0-5 min. after injection of antigen (2) there was an immediate contraction of the gut, followed by a further contraction (at the arrow-head) which was less rapid in develop-

ment and after washing out the bath (after 12 seconds) relaxation was delayed. The first response is due to histamine and is abolished by anti-histamine and the second response is due to S.R.S. and is not abolished by anti-histamine. At (1) is shown a negligible response to 1 ml. of perfusate obtained before injection of 20 mg. egg albumin and at (3) is shown the response to 1 ml. perfusate obtained 5-15 minutes after injection. The immediate contraction ceased at the arrow as with (2) and was followed by the delayed S.R.S. contraction. Four mg. egg albumin (boiled) produced a negligible response of the gut. The unlettered contractions are to 0·2 μg. histamine and at (H) is shown the response to 0·1 μg. histamine. The afterchange in sensitivity of the response of the gut to histamine following treatment with perfusate is well shown.

B.T.Z. Perfusion. While B.T.Z. reduces the response of the gut to histamine this is not the case with the dosage we employ. For example, when B.T.Z., in a concentration of 5 mg. or 10 mg./100 ml., is added to a solution of histamine, 1/10 million, the response of the gut to such a solution is not significantly different from that to a similar solution of histamine without added B.T.Z. This is shown in Fig. 3, where at H is shown the response to 1 ml. of 1:10 million histamine and at B_1 is shown the response to 1 ml. histamine 1:10 million to which B.T.Z. had been added in the concentration of 5 mg./100 ml. and at B_2 is shown the response to 1 ml. 1:10 million histamine to which B.T.Z. had been added in a concentration of 10 mg./100 ml.

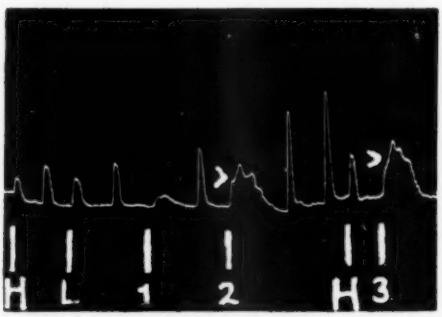

Fig. 2. Responses of the isolated jejunum of a normal guinea pig to histamine and perfusate obtained from a control anaphylactic guinea pig lung. At 1: response to 1 ml. perfusate obtained before injection of antigen; and at 2 and 3: responses to 1 ml. perfusate obtained 0-5 and 5-15 mins. respectively after injection. Perfusate samples left in contact for 12 secs. At the arrow-head the immediate contraction due to histamine ceased. At L: response to 8 mg. lung; at H: response to 0·1 µg. histamine. The unlettered contractions are to 0·2 µg. histamine. Time in mins. Further details in text.

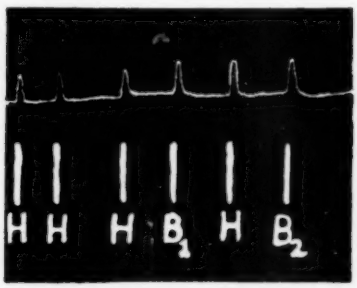

Fig. 3. Responses of the isolated jejunum of a guinea pig to B.T.Z. and histamine. At H: response to 1 ml. histamine 1 in 10 millions. At B1 and B2: response to 1 ml. 1 in 10 millions histamine to which 50 and 100 µg. B.T.Z. have been added. Time in mins.

After the perfusion of the sensitive guinea pig lung with B.T.Z. in Tyrode, in a concentration of 10 mg./100 ml., there is complete inhibition of output of both histamine and S.R.S. following the injection of 20 mg. egg albumin. This is shown in Fig. 4 (Experiment 18), where at (2) and (3) there is a negligible response to 1 ml. of boiled perfusate obtained 0-5 and 5-15 minutes respectively after injection of antigen. What little response there was occurred after a delay of 7 and 4 seconds and these responses were in fact less than that obtained from 1 ml. of boiled perfusate (1) collected before injection of antigen. This response had a latency of 5 sec. and all samples were left in contact with the gut for 16 seconds. This small response to control perfusion fluid is seen from time to time and is due to traces of blood still appearing in the perfusion fluid and, as is seen in the above figure, becomes less as the perfusion proceeds. When small amounts of S.R.S. were present before and after injection of antigen, e.g. Table 1, this was confirmed as S.R.S. and not a slow response from a minute amount of histamine, since the contractions were of the same height both before and after administration of Benadryl in a concentration inhibiting completely histamine effects.

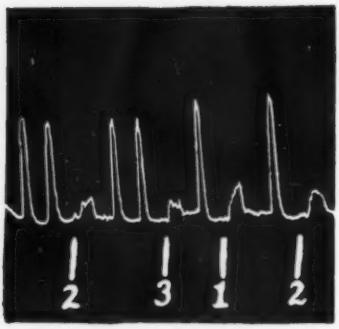

Fig. 4. Responses of the isolated jejunum of the guinea pig to perfusate from anaphylactic guinea pig lung (Exp. 18) perfused with Tyrode containing 10 mg. B.T.Z. per 100 ml. At 1, 2, and 3: responses to perfusate before and after injection of antigen as in Fig. 2. The unlettered contractions are to 0·1 μg. histamine. Time in mins. Further details in text.

The effect of varying the concentration of B.T.Z. on the release of histamine is shown in Fig. 1. In Experiments 17, 18, 19, where 10, 10 and 5 mg. p.c. B.T.Z. respectively were used as the perfusing fluid no estimable output of histamine occurred. In Experiment 25, using 5 mg. p.c. B.T.Z., a trace of histamine was detected in the perfusate. The lung in this instance contained 25 μg. histamine per gm. after perfusion and weighed 3·9 gm. In Experiments 20 and 24, using 2·5 mg. p.c. B.T.Z. in Tyrode, again no histamine was detected in perfusate samples following the injection of antigen. These lungs contained 18 and 8 μg. histamine per gm. respectively after perfusion. In Experiment 21 where 1·25 mg. p.c. B.T.Z. was employed, some histamine (1 in 25 millions) was detected in the first five minute sample after injection of antigen but not thereafter. This lung contained 57 μg. histamine per gm. after perfusion. When the B.T.Z. concentration was reduced to 0·625 mg. p.c. greater quantities of histamine were released (Fig. 1), the concentration being 1 in 16·5 millions in the first five minute sample and 1 in 20 millions in the sample collected five to fifteen minutes after injection.

Inhibition of release of S.R.S. followed the same pattern, some inhibition still being apparent at 1·25 mg. p.c. B.T.Z. (see Table 1). Assay in Experiments 17 and 19 was interfered with by the presence of much blood in samples obtained both before and after injection of antigen.

TABLE 1.
Output of S.R.S.

mg. p.c. B.T.Z.	10	10	5	5	2·5	1·25	0·625	nil	nil
Experiment	17	18	19	25	24	21	23	22	2
0-5′ after injection	* ±	−	* +	−	−	±	+	++	++
10′-15′ after injection	* ±	−	* +	−	−	±	+	+++	++

* perfusate contained much blood
− = nil ± = trace ++ as in 2 and +++ as in 3 Fig. 2.

DISCUSSION.

It is evident that B.T.Z. is a powerful inhibitor of the release of histamine and S.R.S. from the perfused lung of the guinea pig sensitized to crystalline egg albumin. Whereas salicylate is required to be present in a concentration of 25-50 mg. per 100 ml. to produce complete inhibition (Trethewie, 1951b), B.T.Z. is effective in a dose of 2·5-5 mg. per 100 ml. Cortisone and salicylate are equally effective in producing amelioration of the symptoms in early acute rheumatoid arthritis in properly designed trials (M.R.C., 1954, M.R.C., 1955; Bayliss, 1955). The only significant difference between these drugs found in these trials was that the former reduced the sedimentation rate slightly compared with salicylate and the haemoglobin level was higher. Keleman, Majoras, Ivanyi and Kovacs (1950) suggested that salicylates acted through a "stress" pattern releasing cortisone but it was shown (Trethewie, 1951a, b) that salicylates inhibited the release of histamine and S.R.S. in anaphylaxis in the isolated lung in the absence of the pituitary and adrenal gland. It has therefore been averred (Trethewie, 1951a, b; 1954a) that the effect of salicylates in this regard is a peripheral one and is not related to the release of A.C.T.H. and cortisone. It was also suggested (Trethewie, 1954b) that since the response of acute rheumatoid arthritis to salicylate is equal to that of A.C.T.H. if the blood level of salicylate is adequate and that there is no increase in the level of adrenal steroids in the blood (Bayliss and Steinbeck, 1954, further confirmed by Done, Ely and Kelley, 1955), or in the urine (Smith, Gray and Lunnon, 1954), it may then be that the favourable effect of salicylate in acute rheumatoid arthritis is related to an effect on the state of sensitivity or allergy. Detailed studies concerning cortisone in this regard will be published elsewhere. Never-

theless, there are other effects of salicylate, e.g. inhibition of fibrinolysin as described by Ungar, Damgaard and Hummel (1952), and it is to be remembered that Ungar and Mist (1949) relate fibrinolysin activity to allergy.

It would appear significant that the evident anti-allergic effect of B.T.Z. demonstrated here and that of salicylate corresponds to the relative dosage of B.T.Z. and salicylate effective in acute rheumatoid arthritis. The effective maintenance dose of B.T.Z. is one or two hundred mg. daily, while the effective dosage of salicylate is much greater. It is stated that B.T.Z. mounts gradually in the blood owing to slowness of excretion, but unless the daily dose is excreted or metabolised completely within 24 hours, there must be an ever-increasing blood level of B.T.Z. following months of therapy, and a toxic dose would ultimately then accrue in every patient, unless 1-200 mg. were excreted daily with high blood levels of B.T.Z. Blood assay of B.T.Z. is unfortunately not easy so that general agreement is not yet possible on this point. In a certain percentage, therapy is discontinued, owing to water retention or depression of bone marrow function, but in a majority of patients this does not occur.

It is not unlikely that such diversely different drugs as cortisone, B.T.Z. and salicylate are effective in the management of acute rheumatoid arthritis because of the one pharmacological property they all possess in common; namely, that they are all anti-allergic.

SUMMARY.

Butazolidine was shown to inhibit the release of both histamine and S.R.S. from the perfused anaphylactic lung of the guinea pig in a concentration of 2·5-5 mg. per 100 ml.

In this concentration B.T.Z. had no effect on the sensitivity of the guinea pig gut to histamine.

The possible significance of this finding is discussed.

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THE IMMUNOLOGICAL RESPONSE OF FOETAL MICE TO INFLUENZA VIRUS

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Since the introduction of the "self-marker" hypothesis of antibody production by Burnet and Fenner (1949) and the experiments of Billingham, Brent and Medawar (1953) on the production of actively acquired tolerance in mice to skin homografts from a different strain, there has been a great deal of interest in the immunological behaviour of animals exposed *in utero* to a variety of antigens. Burnet and Fenner predicted that an antigen introduced into the circulation of a developing animal before a critical phase in the maturation of the antibody-producing system would come to be recognized as "self". In consequence, the animal's capacity to produce antibody to that antigen would be permanently impaired. Early attempts to produce experimental support for this hypothesis were unsuccessful (Burnet *et al.*, 1950). However, since then, several workers have reported the induction of tolerance to antigens giving rise to classical circulating antibody. Hanan and Oyama (1954) have induced immunological tolerance in rabbits to bovine serum albumen by neonatal injections and this work has since been confirmed and extended (Cinader and Dubert, 1955). Buxton (1954) has reported the induction of tolerance in chicks to Salmonella pullorum by the intravenous injection of a killed vaccine into embryos. Kerr and Robertson (1954) noted that calves given Trichomonas foetus antigen early in life never were able to produce antibody to this agent. However, negative reports have also been quite numerous (Bauer *et al.*, 1956; Burnet *et al.*, 1950; Owen, 1956).

There has been a growing feeling among immunologists that the phenomenon of tolerance can only be produced when the antigen concerned is present in the tissues of the developing animal in large amounts for considerable periods of time. For example, to induce permanent immunological tolerance in rats to foreign erythrocytes, the antigen must be repeatedly injected for about eight weeks (Nossal, 1957). Bearing this in mind, a further attempt was made to induce immunological tolerance in mice to influenza virus. Preliminary experiments, involving the injection of large doses of live virus into the amniotic sacs of mouse embryos, seemed highly promising. Following operation, small num-

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bers of sucklings were born, and allowed to grow up until they were four weeks. They were then challenged with an intraperitoneal injection of virus, and failed completely to produce antibody. However, it soon became apparent that this "tolerance" was an artefact. The technique of operation did not enable all amniotic sacs to be injected, and the survivors represented uninjected mice. The "tolerance" was due to passively transferred antibody derived from the mother's milk neutralizing the antigen injected at four weeks of age. As it was felt that this finding was of considerable significance to workers operating on pregnant animals, the transfer of antibody from mother to progeny, and the effect of passive immunization on the subsequent active immune response were studied in some detail. Subsequently the immunological effect of intrauterine influenzal infections, and of foetal immunization with near-fatal doses of killed virus vaccines, was studied.

Materials and Methods.

Mice. Adult mice of the Hall Institute strain were used for breeding. Females were mated for forty-eight hours and examined for pregnancy 13 days later. The period of gestation was thus known to within ± 24 hour. Suckling mice were routinely weaned at three weeks of age. All mice were given a standard diet of mouse cubes and tap water *ad libitum*.

Virus. The standard A strains PR8 and MEL, and the recombinant strain WS– (Burnet and Lind, 1952) were used. All 3 strains were fully mouse-adapted. Fertile hen's eggs at 11 days' incubation were inoculated suballantoically with a dilute suspension of seed virus kept at – 12° C. The allantoic fluids were harvested 40 hours later, spun at 500 r.p.m. for ten minutes, and stored at 4° C. for up to 48 hours. Dilutions were made as required in physiological saline containing 10 p.c. horse serum (HSS). High titre virus was prepared by spinning the above preparation at 20,000 g. for 20 minutes, and resuspending the resultant pellet in HSS. Killed vaccines were prepared by heating this preparation at 56° C. for 30 minutes.

Virus Haemagglutinin Titrations. These were carried out by preparing serial twofold dilutions of virus in 0·85 p.c. sodium chloride (saline) and adding an equal volume of a suspension of thrice-washed 1 p.c. fowl cells in saline. The conventional partial agglutination pattern was taken as the end-point, and the virus titre was expressed as the reciprocal of the dilution of virus giving partial agglutination (e.g. 500 AD).

Preparation of Sera. If mice were no longer required, they were bled under anaesthesia from the axilla. When serial bleeds were needed, mice were bled from the tail veins. Blood samples were incubated at 37° C. for 45 minutes, centrifuged at 1000 r.p.m. for 10 minutes, and the supernatant serum collected and routinely titrated the same day.

Pre-treatment of sera. To remove non-specific inhibitors of virus haemagglutination, the sera were treated with crude receptor destroying enzyme according to the method of Isaacs and Bozzo (1951). The only modification made was that the period of treatment was reduced from 18 hours to 2 hours. Furthermore, it was found that the cells of most fowls were agglutinated by both normal and immune sera at the starting dilution of one in five. Out of about 80 fowls tested three were found whose cells consistently failed to be agglutinated by mouse sera. These cells were used throughout the experiments.

Antibody titrations. All sera were tested for antihaemagglutinin (AHA) to the immunizing strain. Serial twofold dilutions of sera were made in saline and an equal volume of virus containing fluid of 5 AD titre was added. Finally an equal volume of 1 p.c. fowl cells was added, and the patterns of agglutination read at 40-45 minutes at room temperature.

The reciprocal of the dilution of serum giving inhibition to the level of standard partial agglutination was termed the AHA titre of the serum.

Operations. The technique used was a modification of that used by Billingham, Brent and Medawar (1953).

Pregnant mice from 13 to 19 days gestation were anaesthetized with a mixture of 2 parts ether and one part chloroform. A vertical midline incision, about 1¼ inches long, was made in the skin of the abdomen. The embryos were visualized through the body wall, and were manipulated by finger tip pressure. Virus containing fluids were injected *via* a 27-gauge needle, through the unopened muscular layers of the body wall, either directly into the peritoneal cavities of the embryos, or into the amniotic fluid. In the direct injections, 0·5 p.c. pontamine blue was incorporated into the inocula in order to enable the injected animals to be recognized after birth. The amounts injected varied from 0·01 ml. to 0·05 ml. It was possible to inject about 80 p.c. of the amniotic sacs in each litter. The wound was closed with one layer of interrupted cotton sutures. The miscarriage rate was about 30-40 p.c. and the maternal mortality negligible.

Soluble Haemagglutinin. In one experiment, soluble haemagglutinin was used as a vaccine. This was kindly prepared by Mr. Ada and Miss Perry by a modification of Schaefer's method (Ada and Perry, in prep.). The final HA titre of the preparation was 2400.

Virus content of lungs. The virus content of the lungs of embryos and neonates was determined either by haemagglutination titrations or by egg infectivity. The lungs were dissected free, ground with sterile alundum, and suspended in 0·5 ml. of HSS. Serial dilutions were made in HSS and either the haemagglutinin content or the egg I.D. 50 determined in the conventional manner. If the embryos were less than 17 days old, it was not possible to remove the lungs, and the whole embryo was homogenized.

EXPERIMENTAL.

The first series of experiments deals with transfer of passive immunity.

Placental transmission. A mature female mouse was immunized by two injections of 0·25 ml. of MEL virus, 1000 AD titre, and mated. At eighteen days' pregnancy, the mother was anaesthetized and the infants delivered by Caesarean section. The foetuses were bled from the axilla, and the samples pooled. The mother was similarly bled. The sera were tested for their AHA titre. The titre of the mother's serum was 1500 and that of the infants was 130. From this and several similar experiments it was shown that while some barrier exists to the passage of antibody from mother to foetus, a certain amount can be transmitted, presumably *via* the placental circulation.

Milk transmission. A mouse 14 days pregnant was actively immunized by giving her an intraperitoneal injection of 0·25 ml. of WS— virus, titre 500 AD. When her infants were three days old, they were removed, and a litter of normal infants was substituted. The mice were bled at intervals and the results are summarized in Table 1. From this it is apparent that normal infants can receive large amounts of antibody from an immune foster-mother, and that the antibody can persist for about two months.

Effect of passive immunization from mother on subsequent immune response. Four mice were immunized by an injection of 0·25 ml. of WS— virus, titre 160 AD, at 15 days' pregnancy. Two were allowed to suckle their own infants, while

TABLE 1.

AHA titres of Immune Foster Mother and Sucklings.
Day 0=day of transfer of normal sucklings to immune mother.

Day	5	11	18	28	47	61	67
Mother's Titre	500	500	Discarded				
Infant 1 Titre	180	400	240	60	15	8	<5
Infant 2 Titre	140	280	160	40	8	<5	<5

TABLE 2.

AHA Titre of Sera of Mice Actively Immunized at 15 day gestation, and of their progeny.
Day 0=Day of Birth. Titres are means of groups of 2. Infants transferred on day 3.

Day		3	10	17	25	28 Chall.	37	47 Chall.	55
Immune Mother	Mother	70	120	130	80				
Own Infants	Infant	<5	30	30	10		<5		350
Immune Mother	Mother	60	240	190	50				
Normal Infants	Infant	<5	15	10	<5		<5		900
Normal Mother	Mother	<5	<5	<5	<5				
Infants from Immune Mother	Infant	<5	<5	<5	<5		120		640
Normal Mother									
Normal Infants	Infant				<5		80		900

two were made to suckle normal infants, their own infants being suckled by a normal foster-mother. The mice were bled at weekly intervals, and at four weeks, were challenged with 0·25 ml. of WS— virus, titre of 100 AD. They were bled 10 days later. Ten days after this they were given a booster dose of 0·25 ml. of WS— virus, titre 200, and finally bled eight days after this injection. The results are summarized in Table 2. From this it is clearly shown that the animals suckled by immune mothers have detectable antibody present for over three weeks, and that the animals suckled by normal mothers, even if their own mothers had been immunized, failed to reveal the presence of detectable circulating antibody. The former animals failed to respond to a primary antigenic stimulus at four weeks, but the latter responded normally. After an interval of three more weeks, all animals responded normally.

Quantitation of effect of passive immunity on active immune response. In order to put the findings recorded in the last experiment on a firmer quantitative basis, four weeks old mice were given graded doses of an immune serum prepared by hyperimmunizing a group of adult mice by repeated injections of WS— virus, and testing the response of the recipients to graded doses of antigen given the next day, and to a standard booster injection given 12 days later. Mice were taken in groups of 12 and given 0·5 ml. either of undiluted antiserum,

AHA titre 800, or of serum diluted 1 in 10 and 1 in 50 with saline. Twelve mice were left uninjected. The next day, all mice were bled, and injected intraperitoneally with 0·5 ml. of WS— virus, titre either 1000 AD, 100 AD, or 20 AD. The mice were again bled the next day in order to determine the immediate neutralizing effect of virus on the passively transferred antibody, and then eleven days later. On this day, all mice together with four normal six-weeks old mice were given 0·5 ml. of WS— virus intraperitoneally, titre 250 AD. All mice were bled one week later. The results are summarized in Table 3 where the logarithmic means of the groups of 4 are given.

TABLE 3.

Effect of passive immunity on responses to graded antigen doses. AHA titres represent means of 4 mice.

Titre serum injected	Titre next day	Challenge	1 day	12 days	Challenge	19 days
16 titre ½ cc.	<5	20 AD	<5	<5	250 AD	360
		100 AD	<5	35	250 AD	1100
		1000 AD	<5	60	250 AD	800
80 titre ½ cc.	10	20 AD	9	<5	250 AD	120
		100 AD	6	<5	250 AD	300
		1000 AD	<5	13	250 AD	800
800 titre ½ cc.	115	20 AD	100	20	250 AD	10
		100 AD	80	7	250 AD	<5
		1000 AD	90	6	250 AD	50
Nil serum	<5	20 AD	<5	35	250 AD	1200
		100 AD	<5	190	250 AD	2500
		1000 AD	<5	320	250 AD	2600
		nil			250 AD	960

From these results it can be seen that on the day following the serum injection, the titre of the recipients' serum is about one-sixth of the titre of the original serum. The passively transferred antibody, even if present in undetectable amounts, can markedly reduce the response to an antigenic stimulus given the next day. Large amounts of antibody inhibit secondary as well as primary response. Basically similar results have been obtained by Mason *et al.* (1955) in a different system.

This series of experiments shows that any experiments involving operations on pregnant animals where there is any possibility of immunizing the mother, must be very carefully controlled if evidence of immunological tolerance is to be sought. Foster mothers should be used to rear all infants, and if the transfer of immunity *via* the placenta seems likely a further precaution should be observed. Non-injected litter mates of the foetally injected animals should be used as controls.

The second series of experiments deals with attempts to produce immunological tolerance to influenza virus by various means.

Effects of foetal pulmonary infection. It was hoped that a near-fatal intrauterine pulmonary infection with influenza virus might give the developing antibody-forming system sufficient experience of the virus to induce immunological tolerance. Preliminary experiments showed that undiluted allantoic fluids containing MEL or WS— virus when injected intra-amniotically at any period of gestation lead to multiplication of virus in the lungs and death of the embryos in from 36 to 72 hours. Many animals dying shortly after birth exhibited changes in the lungs which were macroscopically similar to the typical consolidation seen in older animals dying from influenza. The L.D. 50 of MEL virus injected intra-amniotically at 14 days' gestation was approximately determined. Mice were given intra-amniotic injections of graded doses of MEL virus containing 0·5 p.c. pontamine blue. Five days later, the mothers were sacrificed and the uteruses inspected. The number of obviously necrotic foetuses, and of apparently healthy ones was counted. This established that the L.D. 50 of this virus was approximately 0·01 AD titre virus.

Subsequently six mice were taken at 14 days' gestation and the amniotic sacs injected with 0·01 ml. of MEL virus, titre either 0·001 AD, 0·01 AD or 0·1 AD. One mouse was injected with HSS as a control. On the day of birth,

TABLE 4.

AHA titres of mouse sera following intrauterine infection with influenza virus.

Injection (Mel virus)	No surviving to 4 weeks	AHA at 4 weeks	AHA titre 1 week after IPI challenge 200 AD
0·1 AD	4	300 80 20 <5	1400 4000 3000 30
0·01 AD	8	<5 140 16 250 250 400 300 400	50 7000 4000 4000 4500 8000 3000 9000
0·001 AD	13	All <5	240 600 800 <5 250 220 50 280 640 160 80 50 20
HSS No virus	7	All <5	70 70 12 25 160 60 50

all infants were transferred to foster mothers. Thirteen mice in the 0·001 AD group, 8 mice in the 0·01 group and 4 mice in the 0·1 AD group survived to the age of 4 weeks. They were all test bled at the age of four weeks, and then challenged with an intraperitoneal injection of 0·25 ml. of MEL virus, titre 200 AD. They were again bled 8 days later. The results are summarized in Table 4. From this it can be seen, that far from producing tolerance, the intrauterine pulmonary infection has actually immunized many of the mice, and caused an enhanced secondary response in most.

Effects of foetal immunization with high titre vaccine. Subsequent to the successful results obtained in this laboratory in inducing immunological tolerance in rats to foreign erythrocytes (Nossal, 1957), experiments were performed to test the effects of foetal immunization with near fatal doses of heat-killed virus vaccines. This was followed by repeated injections of the same vaccine on the day of birth and twice per week for three weeks. The combined results of these injections were tested by bleeding the animals at four weeks. Preliminary experiments showed that the L.D. 50 of both MEL and PR8 virus vaccines was approximately 0·02 ml. of 5000 A.D. titre virus for 18 day embryos. The results of two typical experiments are recorded in Table 5. In the first experiment, mice at seventeen days' gestation were used, and the embryos injected with 0·02 ml. of 2000 AD titre heat-killed MEL virus, and with 0·05 ml. of the same vaccine on the day of birth and twice per week for three weeks. In the second experiment, 18-day pregnant animals were used and similarly injected with the same doses of PR8 vaccine, 5000 AD titre. In each case, dye was used

TABLE 5.

Serum AHA titres of mice inoculated in utero *and twice per week thereafter with killed influenza virus vaccine.*

	PR8 5000 AD. AHA 4 wks.	MEL 2000 AD. AHA 4 wks.
Treated *in utero*	2800 1600 1600 2400 2000 2000 600 2400	2200 2400 1600 2000 800 2400 2400
Not treated *in utero*, but injected twice per week	2400 1200 2800	800 1600
Not treated *in utero* or after birth	—	<5 <5 <5 <5 <5

to mark the injected animals. In the experiment using MEL virus, some non-injected sucklings were left for four weeks. In both experiments some sucklings not injected *in utero* were also given the injections on the first day of life and twice per week for three weeks. All mice were bled at four weeks of age. From Table 6 it can be clearly seen that the foetal and neonatal immunization did not produce tolerance, but hyperimmunized the mice.

Effect of injection of soluble haemagglutinin. A final attempt was made to design an experiment of the kind used by Hanan and Oyama (1954) in demonstrating tolerance to bovine serum albumen in rabbits. A preparation of soluble haemagglutinin from PR8 virus was made, and the mothers injected intravenously at fourteen days' gestation with 0·01 ml. of 2400 AD titre haemagglutinin. These injections continued daily for 5 days. The doses used must have been near-fatal to the foetuses, because from 2 mothers only six infants were born. These were injected on the first day of life and twice per week thereafter for three weeks with 0·02 ml. of the same preparation. They were bled at four weeks with four normal controls. The mean titre of the treated animals (AHA) was 1500, that of the controls < 4. Once again, hyperimmunization had resulted from the procedures.

DISCUSSION.

One of the findings that prompted the "self-marker" hypothesis of antibody formation was the demonstration by Traub (1938) of the presence in some stocks of mice of lymphocytic choriomeningitis virus in all members of the stock, without the presence of demonstrable antibody. It was postulated that the mice were infected *in utero*, and developed immunological tolerance. This is now accepted as the orthodox view. Despite this, and despite the great current interest in immunological tolerance, no successful experimental attempt at producing the phenomenon of tolerance to a viral agent has appeared in the literature. Furthermore, the two accounts of the phenomenon with other micro-organismal antigens (Buxton, 1954; Kerr and Robertson, 1954) await confirmation. The present series of experiments, together with unpublished experiments done on rats using a series of viral and bacterial antigens (Nossal, unpublished), have shown that under conditions known to produce tolerance to, for example, foreign erythrocytes, no tolerance can be produced to these antigens. In fact, the procedures result in hyperimmunization. It is difficult to escape the conclusion, therefore, that different processes are at work in the maturation of the antibody-forming system, in the two cases. It is clear that tolerance can be obtained for antigens of the type existing on the surface of cells, and if Medawar's interpretation of homograft immunity is correct, for antigens emitted from cell nuclei. The work with bovine serum albumen makes it seem likely that this holds also for purified protein antigens prepared from the serum of other species. However, bacterial and viral antigens may be much more "foreign" in nature to the tissues of

the developing embryo than any of the above cited antigens. The simplest way in which our results can be fitted into the broad framework of the Burnet-Fenner hypothesis is to postulate that antigens such as influenza virus are too "foreign" in nature ever to be mistaken for "self" by the embryo. It can be assumed that antigens of these types injected into foetuses or neonates lie inert in the tissues, subject only to non-specific metabolic destruction, until such time as the animal is mature enough to make classical circulating antibody.

SUMMARY.

Pregnant mice were immunized with influenza virus injections, and it was shown that antibody was transmitted to the foetuses, presumably *via* the placenta, and also to the sucklings through the milk. It was shown that passively transferred immunity could effectively block the response to large doses of antigen.

Foetal mice were exposed to near lethal pulmonary infection with influenza virus, and to intraperitoneal injections of high titre virus vaccines. These procedures resulted, not in immunological tolerance, but in immunization.

The implications of these findings are discussed.

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THE ANALEPTIC ACTIVITY OF BEMEGRIDE TO STRUCTURALLY UNRELATED HYPNOTICS

by A. SHULMAN AND GLENDA M. LAYCOCK

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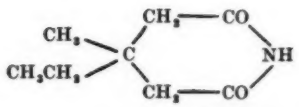
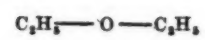
(*Accepted for publication 9th September, 1957.*)

Previous work has indicated the ability of Bemegride, (I) (β-methyl-β-ethylglutarimide, Megimide, NP_{13}), to reverse hypnosis in mice induced by such structurally related hypnotics as the monoureides, barbiturates, thiobarbiturates, glutarimides, diketo-piperidines, diketo-tetrahydropyridines, diketo-thiazanes and diketo-thiazolidines (Shaw, Simon, Cass, Shulman, Anstee and Nelson, 1954; Shulman, 1956; Somers, 1956; Frey, Hushahn and Soehring, 1956; Kimura and Richards, 1957; Shulman and Laycock, 1957a). A suggestion was made that such analeptic activity might be structurally selective and might take place on a specific receptor surface incorporating three suitably disposed sites (Shulman, 1956).

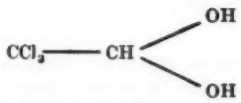
This paper deals qualitatively with the ability of Bemegride[1] to antagonize hypnosis in mice and dogs due to a group of diverse hypnotics not structurally related to Bemegride, namely diethylether (II), chloral hydrate (III), chlorbutol (IV), morphine (V), Oblivon[2] (VI, 3-methyl-pentyn-3-ol), paraldehyde (VII), urethane (VIII) and Viadril[3] (IX, 21-hydroxypregnanedione sodium succinate).



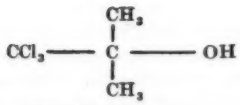
Bemegride (I)
(β-methyl-β-ethylglutarimide)



Diethylether (II)



Chloral hydrate (III)



Chlorbutol (IV)

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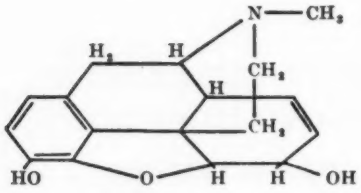
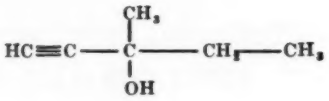
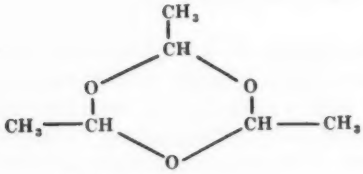
[1] Nicholas Pty. Ltd. (Australia)—"Megimide".
[2] British Schering Research Laboratories Ltd. (England).
[3] Chas. Pfizer & Co. Inc. (U.S.A.).



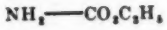
Morphine (V)



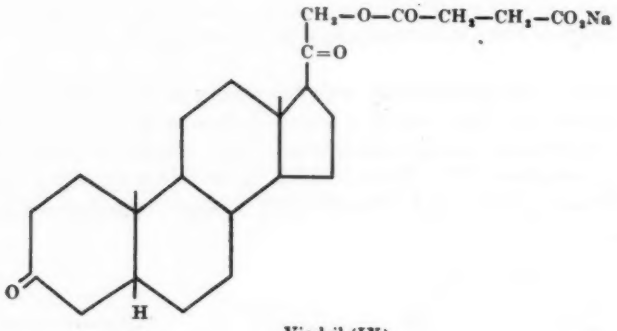
Oblivon (VI)
(3-methyl-pentyn-3-ol)



Paraldehyde (VII)



Urethane (VIII)



Viadril (IX)

(21-hydroxypregnanedione sodium succinate)

EXPERIMENTAL DETAILS.

Analeptic Experiments with Mice.

The following method was used with chloral hydrate, chlorbutol, Oblivon, paraldehyde, urethane and Viadril. Ether and morphine will be considered independently.

Coloured mice of mixed strain and sex weighing between 20 and 40 gm. were divided by random selection into test groups of 20. The analeptic activity of Bemegride was demonstrated by its ability to reduce the sleeping time of mice given a dose of hypnotic so chosen as to give a mean sleeping time of about 60-80 minutes. The sleeping time was considered as the interval elapsing between the loss and the maintained recovery of the righting reflex. All drugs were administered intraperitoneally either in physiological saline

solution (Bemegride, chloral hydrate, paraldehyde, urethane and Viadril) or, when necessary, in a 1 p.c. tragacanth suspension (chlorbutol, Oblivon). Bemegride (20 mg./kg.) or saline was administered at 15 minute intervals.

A "t" test was carried out in all cases where there appeared to be a significant decrease in the sleeping time.

The Interaction of Bemegride with Ether.

Duplicate experiments were conducted using 4 groups of 6 mice. Ether anaesthesia was induced by inhalation; Bemegride was administered intraperitoneally in physiological saline.

Group 1. A proven, highly convulsant and lethal dose of Bemegride (83·3 mg./kg.) ($LD_{50}(I/P) = 27$ mg./kg.; Kimura and Richards, 1957), was administered to each mouse and the effects observed.

Group 2. Each mouse, given Bemegride (83·3 mg./kg.), was permitted to convulse for 15-30 seconds and then placed immediately into an ether-saturated atmosphere and the effects observed. The mouse was then removed from the ether atmosphere until convulsions occurred, put back into the ether atmosphere and this process repeated four times. The time intervals for the onset of Bemegride-induced convulsions, their termination and subsequent recurrence were noted. Finally, the convulsions were terminated by the intraperitoneal administration of paraldehyde (1000 mg./kg.).

Group 3. Each mouse, given Bemegride (83·3 mg./kg.) was placed immediately before the onset of convulsions, into an ether-saturated atmosphere and the effects noted. These mice were subsequently treated in the same manner as Group 2.

Group 4. Bemegride (83·3 mg./kg.) was administered to mice individually maintained under ether anaesthesia and the effects observed over a period of 10 minutes; a second similar injection of Bemegride was then given and the effects again noted.

The mice in Groups 2, 3 and 4 were maintained individually under ether anaesthesia by using a perforated matchbox cover as an anaesthetic mask. This permitted the mice to be kept in a state of moderately deep narcosis, unresponsive to pain, uncyanosed and with a brisk peripheral capillary return as judged by the rapid recovery of pink colouration to the feet.

The Interaction of Bemegride with Morphine.

This was determined in mice and dogs. The dog was chosen because it is the only common experimental animal in which really satisfactory hypnosis, comparable to that in man, is produced by morphine. Both drugs were administered in physiological saline, morphine as the hydrochloride.

(i) *Experiments with Dogs.* Intramuscular morphine (10 mg./kg.) was administered to each of 3 dogs and when they were drowsy and easily manageable (20 minutes), intravenous morphine (10 mg./cc.) was given slowly until each animal was deeply narcotized (immobile and unresponsive to moderately severe pain). The respirations were adequate throughout and there was no cyanosis. Intravenous Bemegride (5 mg./cc.) was then slowly administered and its effects on respiration, response to pain and narcosis observed. Subsequently, the effect of intravenous Bemegride was determined in each unnarcotized dog.

(ii) *Experiments with Mice.* The effect of intraperitoneal morphine (20, 40, 80, 120 and 150 mg./kg.) was determined, in duplicate experiments, in groups of 6 mice of mixed strain and sex (average weight 25 gm.). A proven convulsant and lethal dose of Bemegride (40 mg./kg.) was administered intraperitoneally 5 and 20 minutes after the injection of morphine at each dose level and the effects observed.

RESULTS.

Analeptic Experiments with Mice.

The effect of Bemegride (20 mg./kg.) in relieving hypnosis induced in mice by Oblivon (450 mg./kg.) paraldehyde (1000 mg./kg.), urethane (1000 mg./kg.), chloral hydrate (400 mg./kg.), chlorbutol (250 mg./kg.) and Viadril (125 mg./kg.) is seen in Tables 1 and 2. With the exception of Viadril, Bemegride exhibited marked analeptic activity against these hypnotics and at the dose level employed produced no signs of toxicity except occasionally in the case of urethane.

TABLE 1.

	Oblivon 450 mg./kg.		Paraldehyde 1000 mg./kg.		Urethane 1000 mg./kg.	
Treatment — Saline	+	−	+	−	+	−
Treatment — Bemegride	−	20 mg./kg.	−	20 mg./kg.	−	20 mg./kg.
No. of Animals Taken	20	20	20	20	20	20
No. of Survivors Used in Calculations	20	20	20	20	20	20
Mean Sleeping Time (min.)	69·7	18·0	64·6	10·9	72·2	14·8
Standard Error (S.E.)	7·47		5·62		3·48	
"t" Test vs Saline	6·93***		9·56***		15·11***	

*** = highly significant ($P < 0·001$).

TABLE 2.

	Chloral Hydrate 400 mg./kg.		Chlorbutol 250 mg./kg.		Viadril 125 mg./kg.	
Treatment — Saline	+	−	+	−	+	−
Treatment — Bemegride	−	20 mg./kg.	−	20 mg./kg.	−	20 mg./kg.
No. of Animals Taken	20	20	20	20	20	20
No. of Survivors Used in Calculations	20	20	14	20	20	19
Mean Sleeping Time (min.)	61·8	24·2	68·5	13·4	82·8	61·8
Standard Error (S.E.)	4·61		7·63		4·66	
"t" Test vs Saline	8·15***		7·20***		4·51***	

*** = highly significant ($p < 0·001$).

The Interaction of Bemegride with Ether.

Group 1. All mice convulsed violently and died within 2-4 minutes. The syndrome followed the pattern described by Kimura and Richards (1957).

Groups 2 and 3. Violent convulsions commenced in all animals within ½ to 1 minute and were terminated by ether within 30 seconds. The mice showed no signs of stimulation while under the influence of ether but violent convulsions re-commenced within 2-4 minutes of being taken out of the ether atmosphere and continued until death (2-4 minutes), unless ether was reintroduced. This process was repeated four times without mortality and convulsions were finally terminated by administration of paraldehyde (1000 mg./kg.).

Group 4. No signs of stimulation were observed in the 10-minute interval following the administration of the first dose of Bemegride. The second injection of Bemegride produced a few fleeting tremors in two mice, one of which remained alive for 13 minutes. However, the majority showed no signs of stimulation and died within 2-4 minutes. Thus, the administration of ether prevented or terminated the severe convulsions induced by $3 \times LD_{50}$ of Bemegride. This process of antagonism could be repeated several times without mortality. However, the reverse form of antagonism was not demonstrated under our experimental conditions.

The Interaction of Bemegride with Morphine.

(i) *Experiments with Dogs.* The effect of individual or combined intravenous administration of morphine and Bemegride is seen in Table 3. In the latter case, Bemegride was given immediately the full effects of morphine were evident. The results indicate that, whereas Bemegride (1·5-1·75 mg./kg.) stimulated morphine-induced respiratory depression, there was no definite and lasting antagonism or potentiation of the hypnotic and analgesic activity of morphine.

(ii) *Experiments with Mice.* The effect of individual or combined intraperitoneal administration of morphine and Bemegride is seen in Table 4. Increasing the duration of morphine activity from 5 to 20 minutes produced little alteration in the potency of the effects and negligible difference in the subsequent response of the animals to Bemegride. Morphine produced sluggishness and prolonged periods of immobility in mice without loss of righting reflex. The mice given higher doses of morphine (80-150 mg./kg.) showed also signs of stimulation (Straub tail), while still higher doses (180-330 mg./kg.) often produced convulsive death. The results demonstrate no obvious antagonism or potentiation of any of the measured indices of either morphine or Bemegride activity.

DISCUSSION.

This work has demonstrated the potent analeptic activity of Bemegride in mice to a series of structurally unrelated hypnotics (chloral hydrate, chlorbutol,

TABLE 3.
The interaction of morphine with bemegride in dogs.

Dog		1	2	3
Normal Respiration Rate per Minute		35	28	28
Weight (kg.)		7·0	7·3	7·3
Dose of Morphine Administered (mg./kg.)		37·0	13·0	16·0
State of Animal after Morphine Administration	Hypnosis	Moderately Deep	Moderately Deep	Moderately Deep
	Respiration	22 per minute, deep, irregular	40 per minute, shallow, irregular	30 per minute, shallow, irregular
	Response to Pain	nil	very slight	very slight
	Pupils	constricted	constricted	constricted
Effect of Bemegride on Normal Dog*	Tremors	+(5·0)	+(3·0)	+(2·5)
	Pre-Convulsant State	+(9·0)	+(5·0)	+(5·0)
Effect of Bemegride on Morphinised Dog*	Respiratory Stimulation (depth)	+(1·75)	+(1·5)	+(1·5)
	Tremors	+(3·0)	+(2·0)	+(2·5)
	Pre-Convulsant State	+(5·0)	+(3·5)	+(4·0)

*Figure in brackets = effective does (mg./kg.).

TABLE 4.

The interaction of morphine with bemegride in mice.

Group			1	2	3	4	5	6
Total Number of Mice Used			12	12	12	12	12	24
Dose of Morphine Administered (mg./kg.)			150	120	80	40	20	0
Dose of Bemegride Administered* (mg./kg.)			40	40	40	40	40	40
State of Animals after Administration of Morphine†	Sedation		sluggish, inert, huddled	sluggish, inert, huddled	sluggish, inert, huddled	sluggish	somewhat sluggish	0
	Respiratory Depression		++	++	+	+	−	0
	Response to Pain		−	−	±	+	+	0
	Pupillary Dilatation		+++	+++	++	+	±	0
	Straub Tail		+	+	+	±	−	0
State of Animals after Administration of Bemegride	Onset of Convulsions† (minutes)		1-3	2-4	1-3	1-2	1-2	1-3
	Average Death Time of Group (minutes)	5 minutes after Morphine	10·0	6·5	9·5	8·0	7·5	Range: 3·0-14·0
		20 minutes after Morphine	5·0	5·5	8·5	8·5	7·0	Average: 7·0

* Given 5 or 20 minutes after Morphine.

† No obvious difference at 5 or 20 minutes.

Oblivon, paraldehyde and urethane). Transient signs of stimulation (tremors, Straub tail and mild clonic movements) were observed occasionally in the case of only one hypnotic (urethane) indicating a high order of safety associated with the administration of convulsive doses of Bemegride (20 mg./kg.) under our experimental conditions.

Bemegride was a less potent antagonist to Viadril-induced hypnosis possibly because of the difficulty of displacing the drug from its receptor site owing to the operation of strong van der Waals' forces associated with its large molecular structure. It seems possible that a similar process may strongly influence the potency of Picrotoxin as an analeptic and *d*-tubocurarine as a curariform agent (Dwyer, Gyarfas, Wright and Shulman, 1957).

Bemegride did not provide maintained antagonism to analgesia or hypnosis due to morphine in the dog and mouse, but did reverse morphine-induced respiratory depression in the dog. Similar findings in rabbits have been reported by La Barre, Dumont and Desmarez (1957), who suggested the probable value of Bemegride in cases of clinical respiratory depression of opiate as well as barbiturate origin.

Although ether quickly prevented or terminated the convulsive action of $3 \times LD_{50}$ of Bemegride (83·3 mg./kg.), analeptic activity of Bemegride to ether hypnosis was not demonstrated. However, the high blood concentration of ether required to maintain anaesthesia may make it extremely difficult to detect signs of antagonism with analeptics in doses which do not cause death by a mechanism possibly unrelated to their analeptic activity.

Qualitative evidence has now been presented concerning the analeptic activity of Bemegride in mice to a wide range of structurally related hypnotics (monoureides, barbiturates, thiobarbiturates, glutarimides, diketo-piperidines, diketo-tetrahydropyridines, diketo-thiazanes and diketo-thiazolidines) (Shaw *et al.*, 1954; Shulman, 1956; Somers, 1956; Frey *et al.*, 1956; Kimura and Richards, 1957; Shulman and Laycock, 1957) and structurally unrelated hypnotics (saturated and unsaturated alcohols, aldehydes, carbamates, aliphatic and cyclic ethers, opium alkaloids and sterols) (Frey *et al.*, 1956; Kimura and Richards, 1957; Shulman and Laycock, present work). A quantitative analysis of the analeptic activity of Bemegride in mice to each hypnotic tested qualitatively (and several others) is at present under investigation and the results (Shulman and Laycock, unpublished work) indicate that the analeptic activity of Bemegride to structurally related hypnotics is confined between much narrower limits than that shown to structurally unrelated hypnotics. Bemegride is generally a more potent analeptic to this latter class of substance.

SUMMARY.

Bemegride, which antagonises hypnosis induced in mice by diverse but structurally related hypnotics (monoureides, barbiturates, thiobarbiturates, glutarimides, diketo-piperidines, diketo-tetrahydropyridines, diketo-thiazanes

and diketo-thiazolidines) also reverses the hypnotic activity of a wide range of structurally unrelated hypnotics (saturated and unsaturated alcohols, aldehydes, carbamates, cyclic ethers and sterols).

Ether prevents or rapidly terminates convulsions induced by Bemegride in mice, but the reverse form of antagonism has not been demonstrated. Bemegride antagonises morphine-induced respiratory depression in dogs, but does not reverse analgesia or hypnosis due to morphine in these animals. No obvious signs of antagonism or potentiation between these two substances were observed in mice. Reference has been made to the possible value of Bemegride in the clinical management of respiratory depression of opiate as well as barbiturate origin.

The widespread safety associated with the administration of Bemegride as an analeptic has been indicated and reference made to preliminary quantitative data which support the suggestion of a selective antagonism by Bemegride to hypnotics structurally related to it.

Acknowledgments. We wish to thank British Schering Research Laboratories Ltd. (England), Nicholas Pty. Ltd. (Australia) and Chas. Pfizer & Co. Inc. (U.S.A.) for their generous gifts of drugs used in this investigation.

We are indebted to Mr. K. Healy for technical assistance.

We are most grateful to Dr. K. H. Pausacker for his helpful criticism and to Professor R. D. Wright for his advice and guidance during this investigation.

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THE CHROMATOGRAPHY AND COUNTERCURRENT DISTRIBUTION OF SECRETIN

by J. W. LEGGE, A. S. MORIESON, G. E. ROGERS AND M. A. MARGINSON

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(*Accepted for publication 25th September, 1957.*)

The nature of secretin has been the subject of many investigations since its activity was first recognised by Bayliss and Starling in 1902, but at no stage has it been obtained in pure form.

In view of its physiological importance a re-examination of secretin by the modern methods of peptide chemistry is overdue, and the present paper reports investigations into the homogeneity of active peptides prepared by various procedures.

In the course of this work Jorpes and Mutt (1952, 1953, 1953-4, 1955, 1955-6) have obtained samples more than ten times as potent as those of Mellanby (1926), which, for more than 26 years had been the most potent reported. Our own findings, with reservations due to a different method of bioassay, fully confirm the existence of such potent materials. In addition to the criterion of potency, however, it is now possible to define the product by its distribution coefficient. A fresh approach to questions of homogeneity may thus be made.

Evidence is presented for the existence of at least two forms of secretin, neither as yet homogeneous, in extracts from one species.

EXPERIMENTAL.

Preparations: Several different procedures have been tested during the investigation. Neither Mellanby's procedure (1926) as amended by Harper and Raper (1943) nor a further modification involving the use of a mixture of pig and ox bile salts (private communication from Professor A. A. Harper) was successful in our hands. The further variations of Crick, Harper and Raper (1949) have not been tested.

The Procedure of Greengard and Ivy (1938): This method, referred to below as G & I, has proved extremely reliable. It is given below in a slightly modified form.

Two to three metres of washed, everted, pig intestines, obtained within 15-30 minutes after slaughter, are extracted in 0·15 N HCl for 30 minutes at a temperature of 0-5° C. Four litres of acid are used for twenty pieces of in-

testine. NaCl is now added to a point just short of saturation and a complex mixture of proteins and peptides precipitated.

The salt precipitate is recovered by filtration with Hyflo Supercel, pressed as dry as possible and extracted with 82 p.c. ethanol. This extract is then concentrated with a climbing film evaporator. Sufficient octanol is added to prevent frothing and to ensure that a separate octanol phase remains after the ethanol has been removed; a large amount of contaminating haematin is removed in the octanol phase. The aqueous phase is now adjusted to pH 5·4 and heated to 55° C., an inactive precipitate is removed and active material precipitated by addition of trichloracetic acid to 5·5 p.c. w/v. This precipitate is referred to as S(I).

Further purification is effected by the solution of 1 gm. S(I) in 20 ml. 0·05 N HCl, addition of 80 ml. acetone and precipitation of inactive material by the further addition of 5 ml. of freshly distilled aniline. The supernatant is evaporated to dryness, and further inactive material is removed by treatment with 1-butanol. Active material is dissolved by cautious extraction of the butanol-insoluble residue with successive, small quantities of absolute methanol. Some additional impurity is precipitated from the methanol solution by addition of two volumes of 1-butanol, and the remaining material is precipitated with excess ether and dried. It is now referred to as S(II).

The Procedure of Friedman and Thomas (1950): These workers extract the G & I salt precipitate with methanol, precipitate with acetone, extract impurities from the precipitate with saturated NaCl at 5° C. and then active material with dilute acetic acid, finally precipitating their product with trichloracetic acid.

The Procedure of Gershbein and Krup (1952): The G & I salt precipitate is dehydrated with an excess of acetone and secretin is extracted with ethanol under conditions slightly different from those of Greengard and Ivy (1938). The remaining steps follow the latter procedure except that the pH 5·4 isoelectric precipitation is omitted and an additional precipitation with picric acid is added at the end.

In the earlier stages of the work most of the preparations were carried out by the G & I procedure; the procedures of Friedman and Thomas and of Gershbein and Krup also gave active preparations.

1-*Butanol-trichloracetic Acid Procedure*: This method developed from our counter-current distribution studies. The earliest steps are those of the G & I procedure. The precipitate obtained by salting out the initial extract is made into a slurry with 0·4 volumes of water, and brought to pH 3 with NaOH. Trichloracetic acid is now added to 3 p.c. w/v and peptides extracted with 0·3 volumes 1-butanol. A second and third extract is carried out with 0·25 volumes of 1-butanol containing 2·5 p.c. trichloracetic acid. Centrifugation may be necessary to break emulsions. The three extracts are pooled and washed with 0·6 p.c. aqueous trichloracetic acid. The peptide is now distributed into an

aqueous phase by adding 0·2 volumes of 0·5 p.c. HCl followed by 5 volumes of peroxide-free ether. The organic phase is again extracted with the same volume of HCl, the two extracts are pooled and an acid-insoluble precipitate is removed by centrifugation. The supernatant is now brought to pH 6 with NaOH, a precipitate removed by centrifugation and trichloracetic acid added to a concentration of 5 p.c. After standing 24-48 hours in the cold an oily precipitate is recovered by centrifugation, washed with 5 p.c. trichloracetic acid to remove salt, dehydrated with a 1 : 1 acetone-ether mixture and dried in a desiccator. Successive extractions with small volumes of purified methanol remove a methanol-soluble portion which is then precipitated with ether and dried. The methanol-soluble peptide is then ready for fractionation by countercurrent distribution.

After distribution, the peptide in each tube is recovered in an aqueous phase by addition of ether and HCl. It is stored thus in the cold until assayed. After completion of analysis of the distribution curve suitable tubes are pooled and the peptide recovered. If sufficient peptide is present it may be precipitated by partial neutralisation of the HCl and addition of trichloracetic acid (see above). When the concentration is below 0·2 mg. per ml. it is partially neutralised and extracted into a small volume of 1-butanol with trichloracetic acid. Excess trichloracetic acid and NaCl are removed by washing. The butanol is now dried with anhydrous Na_2SO_4, filtered, and the peptide trichloracetate precipitated by addition of an excess of peroxide-free ether.

Methods.

Bioassay. The procedure was essentially that developed by Ivy, Kloster, Drewyer and Leuth (1930). Cats, rather than dogs, were generally used. After an overnight fast they were anaesthetised with pentobarbital sodium, and the pancreatic duct cannulated. The bile duct was ligated but it was not found necessary to ligate the pylorus. Drops of pancreatic juice, 20-22 per ml., actuated suitable relays and were recorded on a kymograph. Secretin was introduced through a cannula in the femoral vein and washed in with saline.

The amounts of secretin injected were generally chosen so that the response was between 5 and 50 drops, over which region the dose-response relation is approximately linear. Every fourth or fifth dose was that of a provisional standard, linear interpolation adjusting unknowns between these points. The same provisional standard was used for several cats.

This method, while less precise than those in which many animals have been used for each assay, has given results internally consistent to better than 50 p.c. over short periods and to variations no more extreme than a factor of three over the whole investigation. Where critical, samples have been re-assayed using stable standards.

The dose unit used throughout the paper is the cat threshold unit (c.t.u.). It is defined as the amount of secretin which produces a response of 10 drops of pancreatic juice in not more than 10 minutes. It is related to other units used in the literature as follows: 2 cat threshold units = 1 dog threshold unit = 1 clinical unit = 20 Agren cat units (see Greengard and Ivy, 1938; Jorpes and Mutt, 1955-6). Such comparison is only possible if the animals used by various workers have been of approximately equal sensitivity. Based on the responses to a number of samples of G & I, S(II), and to secretin commercially produced by both Wyeth and Lilly, the average sensitivity of our cats has been lower than

that of the animals used by others (Ivy *et al.*, 1930; Greengard and Ivy, 1938; Greengard, Stein and Ivy, 1941). Our results have therefore been adjusted to a 'standard cat' so that they are comparable with those of other workers as well as being internally consistent.

Determination of Peptide Mass: A volume of the peptide solution containing between 5 and 25 μg. peptide was spread on 0·1 N HCl on a surface balance (Alexander, 1947). The force-area curve was plotted over the region where an approximately linear relationship corresponded to the R-group orientation just prior to collapse of the monolayer. The area was measured by extrapolation back to zero force. The usual figure of 1 metre^2 per mg. peptide, confirmed on a number of salt-free samples, was used to convert area to weight.

Routine accuracy of 5-10 p.c. was acceptable in view of the variation in the bioassay. The first determination in a series takes about 15 minutes, including the time to clean and prepare the balance, subsequent determinations less than 10 minutes each. The weight of peptide present in a given sample may thus be determined while the bioassay of the sample is proceeding.

Counter-current Distributions: These were carried out in a 25 tube manual apparatus, constructed according to Lathe and Ruthven (1951). Nylex tubing proved satisfactory for the solvents used. In this apparatus the heavy phase is transferred. Each equilibration was effected by shaking 100 times. A 10-minute period was generally sufficient for the resolution of emulsions. The distributions were conducted in a room at 23 $\pm$ 2° C.

K is defined as the ratio of secretin activity in the organic phase to that in the aqueous phase. It is determined from counter-current distribution data by using the formula $N = nK/K + 1$, where n is the number of transfers and N the tube in which maximal activity is found.

Chemicals: B.D.H. 'Analar' trichloracetic acid (TCA) and B.D.H. 'Laboratory Reagent' dichloracetic acid (DCA) were used. Titration indicated 98-100 p.c. purity in each case.

1-Butanol and 2-butanol were obtained from the Shell Refinery, Altona. No difference in yield of secretin or in its partition coefficient was found after the solvents were further purified. They were therefore used as received.

The large volumes of ether required were redistilled and checked for the absence of peroxides.

Adsorbents: Silica gel was prepared according to Tristram (1946). Where paper was used as support for the aqueous phase during column partition chromatography it was prepared by macerating No. 3 Whatman in a blendor. The paper was first purified according to the procedure of Connell, Dixon and Hanes (1955).

Other adsorbents tried were commercial grades. Before use they were treated with the appropriate solvents under conditions as extreme as those met with during the adsorption-elution cycle of the peptides.

RESULTS.

Adsorbents and adsorption chromatography.

Our experience with adsorbents broadly confirms the conclusions of other workers; elution is difficult and recoveries poor (Greengard, 1948). Oxycellulose and silica gel were the only adsorbents which gave encouraging results. Pectin, which Jorpes and Mutt (1955) found valuable, has not been examined.

The samples of secretin tested were prepared by the G & I procedure, to the S(II) stage, the most potent assaying at 43 c.t.u. per mg. Concentrations of 1 mg. per ml. were adjusted to pH 5 and were gently shaken with oxycellulose (Eastman Kodak '10 p.c. carboxyl') for 16 hours in the cold room. At concentrations of oxycellulose of 1, 3 and 9 mg. per ml., 16 p.c., 49 p.c. and

97 p.c. of the activity, respectively, was removed from the supernatant. On elution with 0·1 N HCl for 4 hours at room temperature, 88 p.c. and 85 p.c. of the adsorbed material was recovered in the second and third of the above cases respectively. Significant changes in potency did not result from the process.

With less potent samples a greater proportion of the activity remained unadsorbed. A comparison of three samples of oxycellulose, reported at 1, 11 and 19 p.c. carboxyl respectively, with the same sample of S(II) used above, indicated that adsorbtion of total peptide varied directly with the degree of oxidation, and that in the first case, impurities were somewhat more readily adsorbed than secretin.

However, since more encouraging results were found with silica gel, experiments with oxycellulose were discontinued. Initial experiments showed that G & I, S(II), could be quantitatively adsorbed on silica gel at pH 6, both from aqueous or from 2 M NaCl solutions. The considerable amount of salt frequently found in S(II) preparations was thus without effect. Secretin remained adsorbed when the gel was washed with water, but could be recovered by elution with dilute HCl. Recoveries of up to 95 p.c. were obtained when elution was effected with 0·1 N HCl in 0·2 M NaCl. Measurement of potency indicated that secretin was more readily adsorbed than inactive peptide, the eluates being found two to three times as potent as the starting material. Chromatography on silica gel was then investigated (c.f. Rogers, 1951).

The material (G & I, S(II)) was run on the column (usually 10 × 1 cm.) at a pH between 5 and 6; some peptide, of low potency, generally passed through. Stepwise elution was then effected with solutions of HCl of increasing strength, ultimately with 0·1 N HCl in 0·2 M NaCl. Bioassay of the eluate fractions indicated the presence of two active components in most of the samples of secretin examined, the first coming off readily between pH 3 and pH 4, and the second with greater difficulty at pH 1 in the presence of salt. An increase in potency of five to tenfold over the material put on the columns was generally achieved.

On five occasions material assaying 160-300 c.t.u. per mg. was obtained, equalling the most potent preparation reported by Mellanby (1926)—250 c.t.u. per mg. At this time Jorpes and Mutt (1953) also obtained material which in our units would assay at 200 c.t.u. per mg.

It proved, however, unexpectedly difficult to develop the above procedures further, although on one occasion chromatography in 90 p.c. ethanol gave some material with a potency of 600 c.t.u. per mg. Even with the largest columns which proved feasible, the recovery of the minute amounts of active peptide from the dilute eluates was inadmissibly low. This fact ruled out the method as a preparative one and even prevented confirmation of the suspected heterogeneity of the preparations by repeated chromatography on successively smaller columns. In an attempt to resolve the question of the heterogeneity of secretin, counter-current distribution procedures were examined.

Counter-current distribution of G & I preparations.

In a series of preliminary experiments partition chromatography of relatively crude, salt-free secretin was carried out on paper strips with a large number of solvent systems. Solvents were removed by dipping the paper in ether and the peptide was stained with solway purple in dilute sulphuric acid. In agreement with what would appear to be the experience of other workers, no system was found which gave better results than those in which 1- or 2-butanol was equilibrated with an aqueous solution of p-toluene sulphonic acid, di- or tri-chloracetic acid.

When the aqueous phase contained mineral acids or ammonia there was no movement of peptides from the origin. With homogeneous systems containing ethanol or propanol, the peptide mixture moved, but no resolution was obtained. When 1- or 2-butanol was used with trichloracetic acid in a concentration of 1 p.c. or greater, the peptides moved with the front without resolution. With 1 p.c. dichloracetic acid or with concentrations of trichloracetic acid or p-toluene sulphonic acid between 0·1 and 0·5 p.c., a number of components could be resolved.

Pilot experiments had shown that the activity of secretin preparations could be recovered unimpaired from such systems by methods referred to earlier. Seven samples were subjected to counter-current distribution. These included different stages of the G & I procedure and a pooled sample from earlier G & I, Friedman and Thomas and Gershbein and Krup preparations. In systems prepared by equilibrating equal volumes of 1-butanol with aqueous trichloracetic acid at concentrations equal to or greater than 1 p.c., little movement of either activity or total mass was observed after 25 transfers. Although of no analytical value, this system is clearly of value in concentrating the peptide from dilute extracts.

Satisfactory resolution was achieved in a system prepared by equilibrating and ageing 2·12 l. water, 1·82 l. 2-butanol, 41 ml. glacial acetic acid and 17·1 ml. dichloracetic acid. This had given adequate resolution on paper and, with crude preparations, separated into its phases more rapidly than when acetic acid was omitted.

Figs. 1 and 2 are typical of the distributions obtained with the other G & I specimens. In all cases tubes at the peak of the distribution curve show an encouraging increase in potency, in one case fortyfold, over the starting product. This is due to a satisfactory separation from inactive material much of which, in the absence of a counter-current apparatus, could probably be removed without too much trouble by distribution on a separating funnel scale. The fact that similar results were obtained either with G & I preparations at the stage of S(I), S(II) or with a picrolonate prepared from S(II) indicates that inactive material does not associate with the hormone during the distribution.

In all cases in which the peptide was given 52 transfers in the above system the maximum potency at the peak of the distribution curve was equal to or

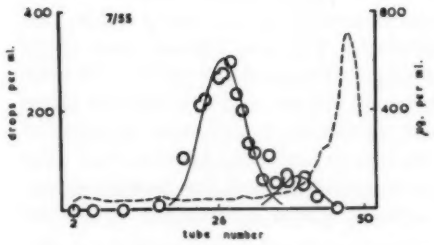

Fig. 1. Counter-current distribution of G & I preparation 7/55. Circles—experimentally determined activities; broken line—peptide concentrations; solid lines—theoretical distributions, major component K = 0·89, minor component K = 0·31.

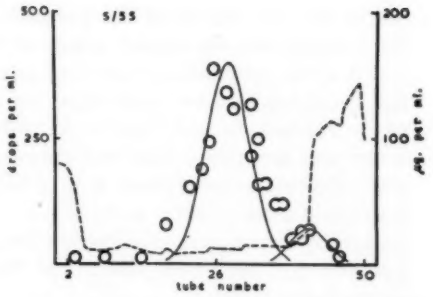

Fig. 2. Counter-current distribution of G & I preparation 5/55. Circles—experimentally determined activities; broken line—peptide concentration; solid lines—theoretical distributions, major component K = 0·82, minor component K = 0·29.

greater than the most active preparations we had obtained by adsorption chromatography. The material in Fig. 2 assayed at 3300 c.t.u. per mg., and is thus approximately ten times as potent as Mellanby's secretin and of the same order as that reported by Jorpes and Mutt (1955-6).

Examination of the curves in Figs. 1 and 2 shows that, in addition to the major component, there is a minor component. The other G & I preparations also showed evidence of this second component. In view of the variability of the bioassay, particular care was taken to establish the existence of the second component, and three animals were used to establish the data in Fig. 2.

The theoretical curves for each distribution have been calculated according to Craig and Craig (1956). The actual distribution coefficients used to calculate the theoretical curves are given in the legends to the figures; in the text, these components will be referred to by average figures, 'K 0·88' and 'K 0·29'.

The minor component, 'K 0·29', travels closely behind the main mass of more hydrophilic, inactive material and it is impossible to draw any conclusions as to its potency. The major peak 'K 0·88' appears to correspond to one component but the potency varies over the course of the activity curve. Inactive peptide is therefore still present.

First modification of the preparation of secretin.

The above experiments suggested certain possible simplifications in the preparation. Sodium chloride has been used in large amounts in most of the preparations since its introduction by Weaver, Luckhardt and Koch (1926). Our own experience, as well as that of other workers, has been that its removal presents certain difficulties. In the 1-butanol-2·5 p.c. aqueous trichloracetic acid system the partition coefficient (activity per ml. organic phase/activity per ml. aqueous phase) is greater than 24. This suggested that the initial salting

out of the acid extract of the intestines might be avoided if direct extraction of the secretin into the organic phase of the above system proved feasible.

A small preparation from dog intestine showed that this was possible, and five preparations were made from pig intestines. The acid extracts were made at the abattoir, brought back to the laboratory and adjusted to pH 3. Trichloracetic acid was then added to a concentration of 2·5 p.c. and the aqueous phase was extracted several times with 1-butanol containing appropriate amounts of water and trichloracetic acid (2 p.c.). Emulsions were troublesome but were broken by centrifugation. The butanol solution was washed, the peptide driven into 0·5 p.c. HCl with ether, and the aqueous phase centrifuged to get rid of insoluble material.

After partial neutralisation of the supernatant HCl a precipitate was obtained by addition of trichloracetic acid to a concentration of 5 p.c. w/v. This product was examined by counter-current distribution in the 2-butanol-acetic acid-dichloracetic acid-water system used successfully with the G & I preparations.

To our surpise, the main product appeared in all cases to have solubility characteristics which differed from the G & I products illustrated in Figs. 1 and 2. After 25 transfers the activity curve of preparation 12/55 showed unequivocally (Fig. 3) that two components were present.

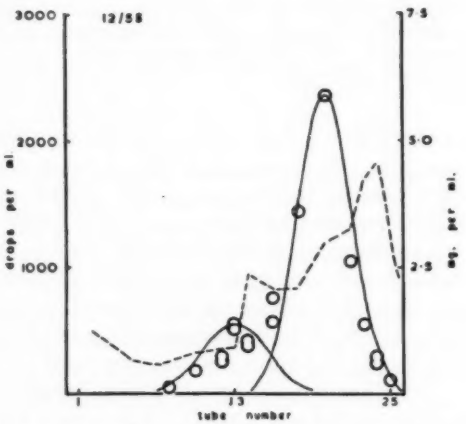

Fig. 3. Counter-current distribution of secretin prepared according to the first modification 12/55. Circles – experimentally determined activities; broken line–peptide concentration; solid lines – theoretical distributions, minor component K = 1·0, major component K = 0·27.

Tubes 10-15 and 16-22 were therefore withdrawn and concentrated. Each was given a further 50 transfers under the same conditions. The 'K 0·88' component in Fig. 3, comprising one-sixth of the total activity, was found, after its second distribution, to have moved into the same position as the major, 'K 0·29', component. The activity remaining in the original position was barely detectable.

The major peak did not alter its position after redistribution (Fig. 5). A small amount of the 'K 0·88' component was present but no more than would have been expected from the overlap of the two peaks in Fig. 3. All the activity in the first distribution was recovered in the second and third. After 25 transfers each of the other four preparations carried out by this method gave evidence

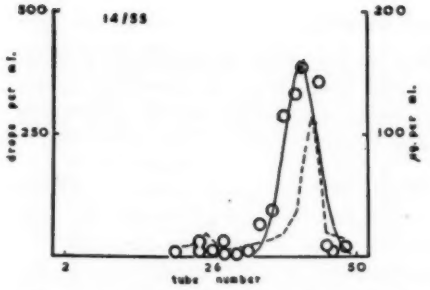

Fig. 4. Counter-current distribution 14/55, minor component from distribution illustrated in Fig. 3. Circles—experimentally determined activities; broken line—peptide concentration; solid line—theoretical distribution for K = 0·29.

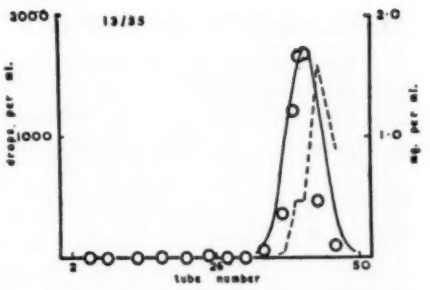

Fig. 5. Counter-current distribution 13/55, major component from distribution illustrated in Fig. 3. Circles—experimentally determined activities; broken line—peptide concentration; solid line—theoretical distribution for K = 0.29.

of two components either by a significant though small peak, or by a skewness in the expected direction.

The maximum potency observed in this series of experiments was 1500 c.t.u. per mg. as against the maximum of 3300 c.t.u. per mg. found after the counter-current distribution of the G & I secretin. The separation of active, from inactive, faster-running material is not as efficient as in the case of the G & I preparations since the relevant K's are closer together. The main peak appears to represent one secretin but other peptides are present since once again there is no evidence of constant potency over the curve.

The two components present in Fig. 3 have approximately the same partition coefficients as the two components observed in secretin prepared by the G & I procedure, but they are present in different amounts in the two cases. The simplest hypothesis is that the 'K 0·88' component may be transformed into the 'K 0·29' component and that conditions favouring this change have been accidentally revealed by the modified preparation.

A re-examination of the details of the preparations showed that the most obvious differences which might have been responsible for the above phenomenon lay in the omission of salt and in the fact that the initial acid extract stood for 5-6 hours before trichloracetic acid was added. This step was taken when the extract had been brought back to the laboratory and its temperature had approached that of the surroundings. Such conditions may have been favourable for enzymatic action, particularly as the extracts were made from a region of the intestine rich in proteolytic enzymes. It was thought unlikely that the addition of salt had any direct effect on secretin, or that it caused significant alterations in its extractability by the organic phase, but rather that its effect may have been to precipitate proteins and retard any enzymatic action. In the earlier G & I procedure salt was added before the temperature could have risen much above 5-10° C.

Further experiments have clarified certain aspects of the above problem.

Recommended 1-butanol-trichloracetic acid method.

The details of this procedure have been set out in the experimental section. Scrupulous attention was paid to the time and temperature of extraction of the intestines, NaCl being added precisely 30 minutes after the last of each batch of 20 intestines had been placed in the acid.

The distribution curve of the secretin obtained in preparation AP2/56 is illustrated in Fig. 6.

It can be seen that the activity is represented almost wholly as the 'K 0·88' component. In three preparations the maximum potency varied from 900-1100 c.t.u. per mg. In all cases the potency varied over the main peak so other peptides are present.

This result shows that material resembling the main G & I component may be obtained by the modified procedure.

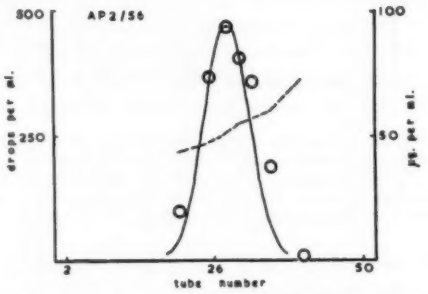

Fig. 6. Counter-current distribution of secretin prepared according to the recommended 1-butanol-trichloracetic acid method AP2/56. Circles—experimentally determined activities; broken line—peptide concentration; solid line—theoretical distribution for K = 0·82.

Measures aimed at diminishing enzyme action.

Further experiments were carried out to gain more information on the conditions which may determine the amount of the more hydrophilic secretin formed. On each of two occasions, the intestines from a large batch of pigs were processed by four different methods; in A1/57 they had been fed barley, and in A2/57, wheat, in the 48 hours prior to slaughter.

(a) Control preparations. The intestines were extracted for 30 minutes at a temperature of 5° C. Salt was then added to the extract and the remaining steps carried out according to the same 1-butanol-trichloracetic acid procedure used in AP2/56.

(b) The intestines were extracted for 30 minutes, the extract held at a temperature of 5° C. or below for 24 hours and the secretin extracted directly from acid with trichloracetic acid and 1-butanol without the use of salt. Subsequent manipulations followed (a).

(c) The intestines were extracted for 30 minutes at a temperature of 5° C. The extract was then brought rapidly to 95° C. with steam, and cooled to a temperature of 20° C. before extracting the secretin as in (b) with butanol and trichloracetic acid without the use of salt. Subsequent manipulations of the extract followed (a).

(d) The intestines, suspended in a little water, were brought rapidly to 95° C. with steam, and, after standing for 10 minutes, were cooled as rapidly as

possible. They were then extracted for 30 minutes in acid, which was worked up without the use of salt, as in (b) and (c).

In cases (b), (c) and (d), alkali, sufficient to neutralise the HCl present, was added immediately before extraction with butanol so that the conditions of extraction were comparable with (a).

In procedure (c), A1/57, the pH was adjusted to between 4 and 5 before heating, in order to duplicate the original Bayliss and Starling (1902) preparation. In A2/57 the heating took place at a pH of approximately 3.

The eight samples of secretin were all subjected to counter-current distribution, amounts of the phases being taken so that the concentration of peptide was roughly comparable in each case. Twenty-six transfers were made, the results being recorded in Table 1 as the ratio of the activity at tube 20, to that at tube 14. This ratio has been taken as an approximate measure of the proportions of the two components, 'K 0·29' and 'K 0·88' respectively.

TABLE 1.

The effect of variations in the preparations on the proportions of the two components.

	Activity $\frac{\text{Tube 20}}{\text{Tube 14}}$	
	A1/57	A2/57
(a) Control preparations	0·20	0·30
(b) Extract held for 24 hours in cold. Salting out step omitted	0·40	0·37
(c) Extract heated to 95°C. Salting out step omitted.	1·0	0·50
(d) Intestines heated to 95°C. before extractions. Salting out step omitted.	0·25	0·24

It can be seen that the addition of salt, or the heating of the intestines before extraction gave a smaller proportion of the hydrophilic component than in case (b) when the extracts were allowed to stand overnight in the cold. The fact that the greatest amount of the hydrophilic component is found in case (c) when the extracts are heated either at pH 3 or pH 5, may reflect the enzymatic action during the period required to bring approximately 2 kg. of intestines suspended in water to a temperature at which denaturation might be expected.

In view of the labour involved it was not possible to measure peptide mass in this series of counter-current distributions and no information is thus available as to the potency of the secretin. It is of interest that approximately twice as much secretin activity was recovered per length of intestine when the intestines were heated before extraction, case (d), as in the standard procedure recommended. Further experiments are required before it can be ascertained

whether this is due to inhibition of enzymic action or to better extraction. Likewise, additional experiments are required in order to determine if this observation should be incorporated in an amended preparation. The operation of heating and cooling the intestines is an inconvenient one, and the acid extraction of the heated intestine introduces much additional inactive protein, the removal of which may well be more trouble than the extra amount of secretin extracted is worth.

The disappearance of secretin on storage of crude extracts of intestine has been known for a long time. The original acid extracts may be shown to dissolve fibrin strands in a few hours at room temperature, but a suspension of the salt precipitate in dilute acid saturated with NaCl does not. If, as appears possible, the enzymic agent is pepsin, it is of interest that Northrop, Kunitz and Herriot (1948) report pepsin to be quite soluble in 70 p.c. alcohol, and it might thus be extracted with secretin in the second step of the G & I procedure. It was thought possible that the transformation of the 'K 0·88' component to the 'K 0·29' component (Fig. 4) might have been due to some pepsin travelling in this region in the distribution. Measurement of the partition coefficient of a sample of B.D.H. 1/2500 pepsin, however, showed it to be wholly in favour of the aqueous phase and it would not be expected to follow the secretin into the first butanol-trichloracetic acid extract. We are therefore not able to present a satisfactory explanation for the observations in Fig. 4.

The applicability of other preparative procedures.

Several attempts have been made to resolve partially purified samples of secretin by column partition chromatography. The aqueous phase has been adsorbed on Hyflo Supercel, on Whatman cellulose powder and on disintegrated Whatman No. 3 paper (see Methods). The first and third of these proved satisfactory, recovery of activity being as good as with counter-current distribution. Our primary interest was in their use for preparative purposes. For this, however, they proved less satisfactory than counter-current distribution. They might well prove more satisfactory for analysis. Reverse phase partition chromatography has not yet been examined.

Purified preparations have also been examined by means of paper chromatography. The systems referred to earlier have been used, both with and without pretreatment of the paper with the nonvolatile components. Several spots may be distinguished when the most potent samples are examined, and activity may be recovered from the paper by elution with 0·1 N HCl saturated with 1-butanol. However, no change in potency has been observed in the eluted material and it appears likely that the other components observed are artefacts or degradation products.

Finally, encouraging results have been obtained by starch gel electrophoresis, carried out according to Smithies (1955). Several components have been observed in highly purified material and a recovery of 50 p.c. of the activity has been achieved.

DISCUSSION.

Although several of the samples reported in the present work would appear to be almost homogeneous with respect to their biological activity, none has been obtained free from inactive peptide. We think it improbable, however, that specimens with potencies of 3300 c.t.u. per mg. are grossly admixed with such inactive material. Material one-third as potent has been obtained on many occasions, and since several other procedures, of high discriminatory powers, have been shown to be applicable, the goal of a rigorously purified product would appear in sight.

We have been able to compare a number of reported procedures for the preparation of secretin and have found that the method we suggest is more convenient and gives more potent material in comparable yield. We have not, however, compared it in the laboratory with the briefly reported method of Jorpes and Mutt (1955-6) by which equally potent material has been prepared.

In that two components have been recognised by their partition coefficients, the method may be superior to others, since it adds an additional characteristic to those which secretin has hitherto been shown to possess.

A further advantage would seem to attach to the fact that the use of phase separations enables the methods to be scaled up or down at will, and small amounts of hormone to be recovered from large amounts of solution. It may now be possible to reinvestigate a number of puzzling features associated with the synthesis of secretin in the intestine. In common with other workers, we have observed variations in yield which would seem to be outside the limits set by intrinsic variability of any particular method, and which may be related either to the dietary history of the animals or to their physiological condition before slaughter. Preliminary experiments in which attempts have been made to select animals with a known history and to control their diet immediately before slaughter have, so far, been inconclusive.

SUMMARY.

Modifications of the preparation of secretin from pig intestines are reported which have certain advantages over published procedures.

Studies on the counter-current distribution of this product and of secretin prepared by other methods have shown that at least two active forms of the hormone may be distinguished. One is thought capable of transformation into the other.

Although neither of the two forms of secretin can be considered homogeneous, both have been regularly obtained with potencies in excess of 1000 c.t.u. per mg. and one form occasionally with a potency as high as 3000 c.t.u. per mg. They are thus of the same order of potency as any hitherto reported.

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ELECTRON MICROSCOPY OF THE AMPHIBIAN RENAL GLOMERULUS

by R. K. F. PAK POY

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Work in this laboratory has latterly been concerned with the electron microscopic study of the morphology of the vertebrate renal glomerulus and at present the structure of the avian glomerulus (Pak Poy and Robertson, 1957) and the marsupial glomerulus (Pak Poy, 1957) have been reported. The structure of the glomeruli of higher mammals has already been reported by numerous workers (Hall, 1953; Hall, Roth and Johnson, 1953; Reid, 1954; Mueller, Mason and Stout, 1955; Yamada, 1955; Pease, 1955; Rinehart, 1955). The present paper describes the structure of the amphibian mesonephric glomerulus in the toad (*Bufo marinus*) and in the frog (*Lemnodynastes tasmaniensis*). Earlier workers (Sedgwick, 1905; Noble, 1954) have described the amphibian renal glomerulus as a knot of vessels projecting into the dilated end of a tubule and did not mention any distinguishing characteristics. More recently Fawcett (1954) studied the frog's renal glomerulus with the aid of an electron microscope and his brief description of this structure is essentially similar to that described for the metanephric glomerulus of mammals.

Materials and Methods.

Animals (of unknown age) were pithed and the kidney exposed by an antero-lateral incision. Osmium tetroxide (1 p.c.) at pH = 7·4 buffered with acetate-veronal (Palade, 1952) was dropped onto the surface for ten minutes and a piece of renal cortex was removed, sliced into 1 mm. cubes and placed in the above fixative for 1 to 4 hours at 4° C. After fixation the tissue blocks were washed in distilled water, dehydrated with alcohol, impregnated with pure methacrylate and embedded in 20 p.c. methyl methacrylate in butyl methacrylate. Thin sections were cut on glass knives in a thermal-expanding microtome, mounted on parlodian-covered grids and were examined in either a Metropolitan-Vickers EM4 electron microscope at 50 k.v. using a 100 μ objective aperture or a Phillips EM100 electron microscope. Osmium tetroxide (1 p.c.) buffered with phosphate to a pH of 7·4 and isotonic with frog's blood (Glasser, 1944) was also used as a fixative.

RESULTS.

Glomerular loop. The capillary loops are as numerous and as tortuous as those described in the glomeruli of higher mammals. A major portion of the capillary lumen is lined by a thin sheet of endothelium similar to that described

in higher mammalian glomeruli (Hall, 1953; Yamada, 1955; Pease, 1955; Rhodin, 1955), in the avian glomerulus (Pak Poy and Robertson, 1957) and the marsupial glomerulus (Pak Poy, 1957). The remainder of the lumen is lined by the main bulk of endothelial cytoplasm in which the nucleus of the cell is situated. The endothelial sheet is not so uniformly thin as in the mammal, for throughout the endothelial lining are increased amounts of cytoplasm which project not only into the capillary lumen but also into the basement membrane (Fig. 4). "Pores" similar to those described in mammalian glomeruli (Yamada, 1955; Pease, 1955; Rhodin, 1955) and in the avian glomeruli (Pak Poy and Robertson, 1957) are present in the thin endothelial lining but they are not as numerous as in the mammalian glomeruli although slightly more numerous than in the avian glomerulus (Fig. 2). The main body of the cell with its nucleus is situated at the periphery of the capillary loop as frequently as it is situated against the intercapillary tissue or the main bulk of another endothelial cell. The nuclei are generally ovoid and the cytoplasm contains mitochondria, rather scanty endoplasmic reticulum and dense granules.

Epithelial cells. These cells lie free in Bowman's space and possess large ovoid or round nuclei which may be indented and may contain nucleoli. The cytoplasm contains mitochondria, endoplasmic reticulum and dense granules. The epithelial cells of the amphibian glomerulus differ from those described in the mammalian and the avian glomeruli in two respects; firstly the main body of the cell may lie flat against the capillary loop basement membrane and secondly the pedicellar arrangement is not as complex. The terminal foot processes are generally shorter and wider than those described in the mammalian and avian glomeruli, although the elongated filamentous form does exist. Some of these processes are small and appear like a chain of droplets along the surface of the capillary loop basement membrane, while others are larger and "blob"-like (Fig. 4), and tend to spread as a flat layer over the basement membrane. Another type of process takes the form of a flat layer of cytoplasm lying free in Bowman's space with small projections resting on the capillary loop basement membrane. Sometimes these terminal projections are absent and the layer of cytoplasm lies flat on the basement membrane (Fig. 1) as has been described by Farquhar, Vernier and Good (1957), as occurring in lipoid nephrosis. The intricate pattern formed by interdigitating processes described in mammalian and avian glomeruli was not noted.

Basement membrane of capillary loop. The structure of the basement membrane of the capillary loops differs from that described in the mammalian glomeruli by Pease (1955), Yamada (1955), Rhodin (1955), and in the avian glomerulus by Pak Pay and Robertson (1957), and in the marsupial glomerulus by Pak Poy (1957). In some sections it is a dense homogeneous layer as described in the mammalian glomerulus by Hall (1953), and Reid (1954), and in the frog's glomerulus by Fawcett (1954), and it has the same electron density

as the intercellular material of the intercapillary tissue and is continuous with it. However, in other sections there is a thin dense layer which is separated from the pedicellar cytoplasmic membrane by a thin less dense layer (Fig. 3). This thin dense layer and the less dense layer are continuous around the surface of the intercapillary tissue to the next capillary loop and at the hilum the thin dense layer is continuous with Bowman's capsular basement membrane. The rest of the basement membrane on the endothelial side of this dense layer is composed of a spongy moderately dense material which may vary in width from 1000A to 4,500A, and is continuous with the intercellular material of the intercapillary tissue. There is no thin dense layer adjacent to the endothelial lining.

Intercapillary tissue. In almost every section through a glomerulus there are cells and cytoplasmic projections situated between capillary loops in the plane of sectioning (Fig. 8). Even sections through the peripheral parts of adjacent capillary loops show cells between them (Fig. 7). In plane of section these cells and cytoplasmic projections are separated by endothelial cells or endothelial cytoplasm from direct contact with blood and from Bowman's space by the thin dense sub-epithelial layer of the capillary loop's basement membrane. Similar intercapillary cells have been described in higher mammalian glomeruli by Yamada (1955), Policard *et al.* (1955), and in the avian glomerulus by Pak Poy and Robertson (1957), and in the marsupial glomerulus by Pak Poy (1957).

Pericapillary tissue. In the space between epithelial pedicels and the endothelial lining of the glomerular capillary loop in which in mammals and birds there is usually only basement membrane, there are in the amphibian glomerulus projections of cytoplasm and cells (Fig. 5). These cells are situated between the endothelium and the dense sub-epithelial layer of the basement membrane in the spongy moderately dense material. The space between these cells and the endothelium is filled with the spongy moderately dense material with no formation of a dense layer. These peri-capillary cells have ovoid nuclei and the cytoplasm contains the usual organelles such as mitochondria, endoplasmic reticulum and dense granules. There are no features to distinguish them from endothelial cells or intercapillary cells except their positions.

Bowman's capsule. Bowman's capsule is lined by a layer of flattened epithelial cells which contain flattened nuclei which may be lobulated. The cytoplasmic membrane rests on a thin dense layer similar to that seen beneath the epithelial pedicels. If a tubule is adjacent to Bowman's capsule there is a thin dense layer beneath the cells of the tubules and this is separated by less dense spongy intercellular material from the dense layer beneath Bowman's capsular cells (Fig. 6). Millen and Rogers (1956) have described a similar arrangement in the choroid plexus between the endothelial and the epithelial cells and have stated that when these cells are very close and no intercellular space exists the two dense layers come into contact and then it appears as the basement membrane of glomerular capillaries.

Fig. 1. The micrograph shows two epithelial cells lying free in Bowman's space. Their nuclei are designated (v) and their cytoplasm (z) containing mitochondria (m). The cell on the left side gives rise to two pedicels (p) lying flat on the capillary loop basement membrane. The thin endothelial sheet (es) lines the capillary lumens (c). An "intercapillary" cell is at (k) and cytoplasmic projection at (w). × 14,000.

Fig. 2. The micrograph shows a capillary lumen (c) lined by a thin endothelial sheet perforated by "pores" (arrows). Epithelial pedicels are represented at (p). × 16,000.

Fig. 3. The micrograph shows the wall of a capillary, the lumen of which is at (c). The endothelial cell nucleus is at (n) and the thin endothelial sheet at (es). The sub-epithelial basement membrane is indicated by (b) and the peri-capillary space (o) is filled with a spongy moderately dense material. Epithelial pedicels are represented at (p). × 20,000.

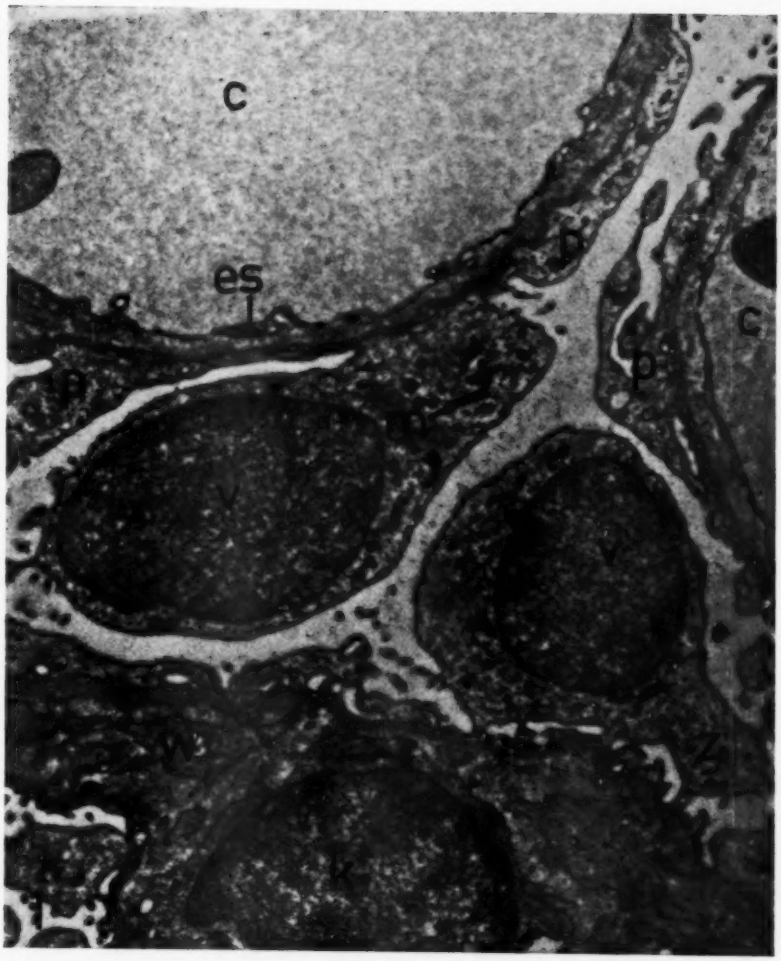
Fig. 4. The micrograph shows a cytoplasmic projection (x) in the peri-capillary space (o) at the periphery of a capillary loop, the lumen of which is at (c) and the endothelial lining at (es). A "blob"-like pedicel is at (p). × 16,000.

Fig. 5. The micrograph shows a peri-capillary cell with its nucleus (h) and cytoplasm (a), and also a peri-capillary cytoplasmic projection (x). An endothelial cell (n) lines the capillary lumen (c) containing red blood cells (rbc). Epithelial pedicels are represented at (p). Notice the absence of a sub-epithelial basement membrane and the presence of a moderately dense material in the intercellular space. × 11,000.

Fig. 6. The micrograph shows Bowman's capsular cell (g) resting on dense layer (d). A tubular cell (q) with mitochondria (m) in its cytoplasm rests on a dense layer (t) with less dense intercellular substance between the two dense layers (t) and (d). Bowman's space is represented at (y). × 12,000.

Fig. 7. The micrograph shows the presence of "intercapillary" tissue (k), (w) at the periphery of a glomerulus. Bowman's capsular cell is at (g). A red cell (rbc) is present in the capillary lumen (c) on the left. The thin endothelial sheet (es) separates the intercapillary cell (k) from the capillary lumen (c) on the right side. A pedicel is present at (p). × 7,000.

Fig. 8. The micrograph shows two capillary lumens (c) with "intercapillary" cells between them. The nuclei of the "intercapillary" cells are at (k) and their cytoplasm at (w). Mitochondria (m) are present in the cytoplasm. In Bowman's space on the left side are pedicels (p) and on the right side an epithelial cell nucleus (v) with its cytoplasm (z). The thin dense sub-epithelial basement membrane (b) can be seen passing from the capillary around the intercapillary tissue. The lumen (c) of the upper capillary is lined by an endothelial cell (n) and endothelial sheet (es) and also contains a red blood cell (rbc). × 13,000.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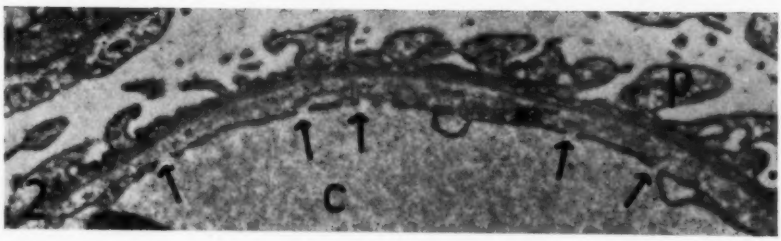
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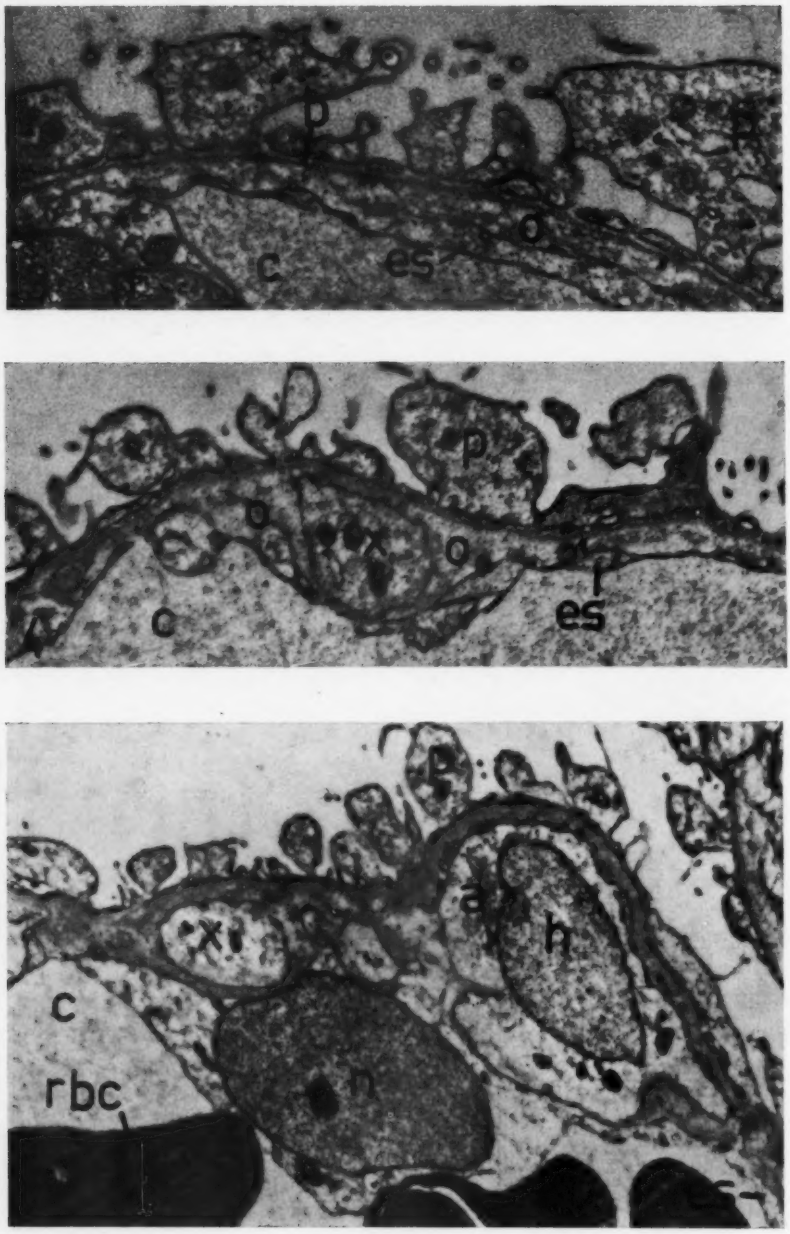
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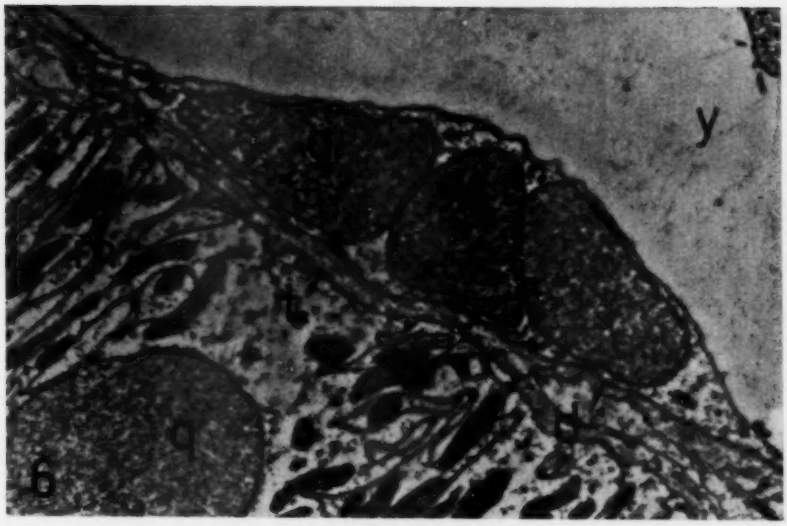
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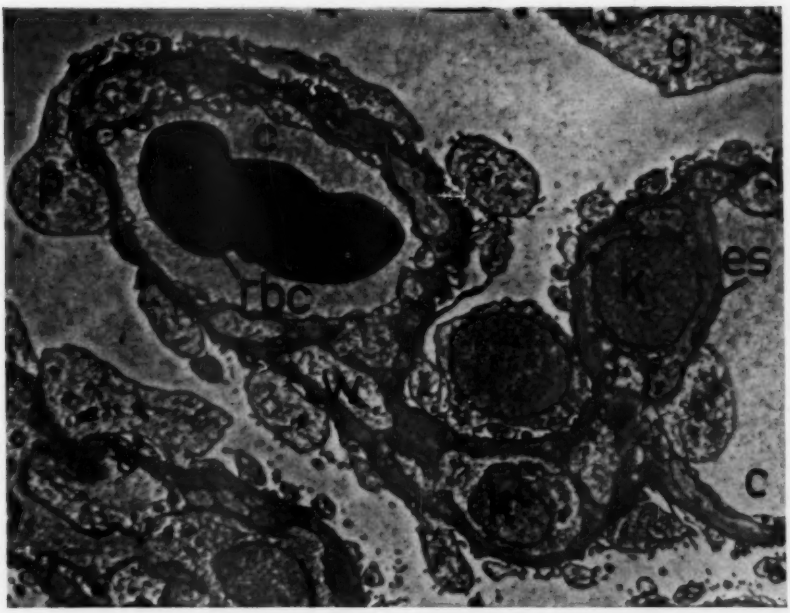
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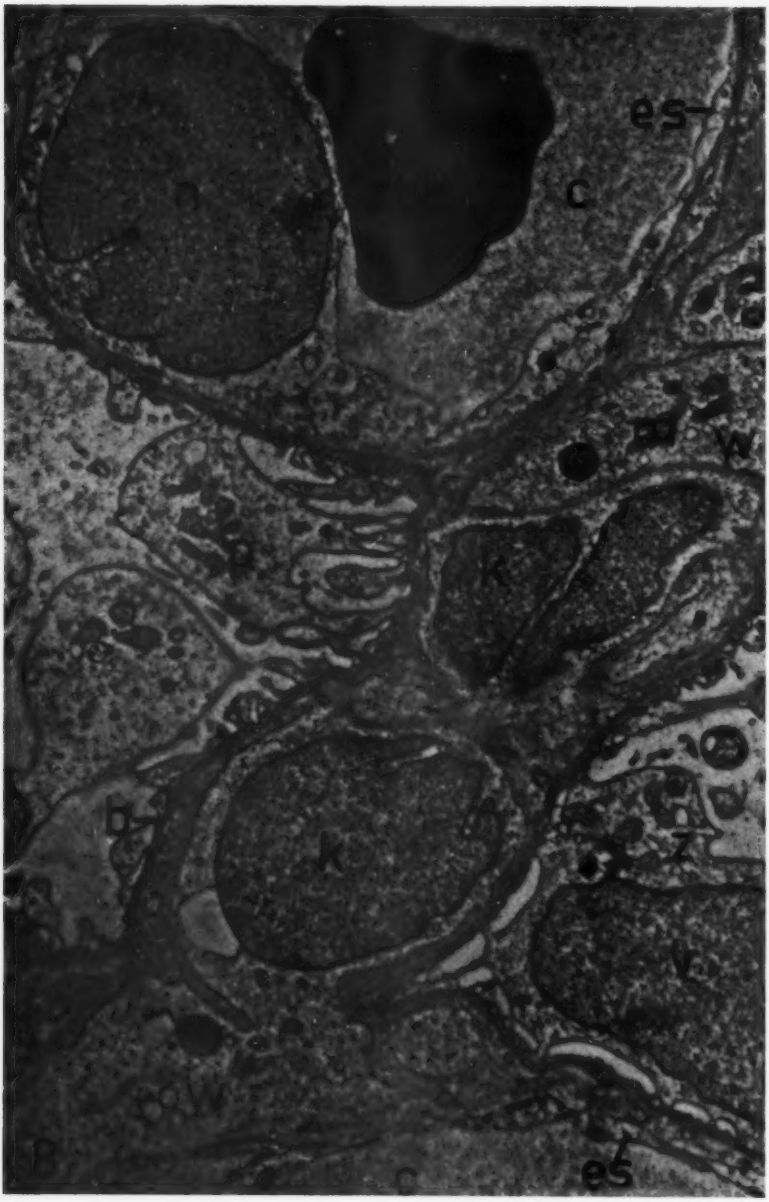
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Hilum. At the hilum there is direct continuity between the thin dense layer of Bowman's capsule and the thin dense layer below the epithelial cells. The endothelial cells of the arterioles were separated from the remainder of the wall by moderately dense intercellular material.

DISCUSSION.

The presence and nature of endothelial pores and intercapillary tissue have been discussed at great length by numerous workers (Hall, 1953; Mueller, Mason and Stout, 1955; Yamada, 1955; Policard, Collet, Giltraire-Ralyte, 1955; Allen, 1951; Bell, 1950; McManus, 1948) and the present discussion will be limited to the two interesting findings in the mesonephric renal glomerulus, *viz.* the structure of the capillary loop basement membrane and the presence of a peri-capillary space containing peri-capillary cells.

The structure of the capillary loop basement membrane provides some interesting features for comparison with basement membranes described in electron microscopic studies of other organs. Millen and Rogers (1956) and Maxwell and Pease (1956) described the basement membrane in the choroid plexus as a thin dense layer beneath the endothelium and also beneath the epithelium. Dempsey and Wislocki (1956) reported a similar structure in the cat's placenta, and Ekholm and Sjostrand (1957) described a similar dense layer beneath the glandular cells of the thyroid. It may therefore be justifiable to conclude that the thin dense layer beneath the epithelial pedicels is a sub-epithelial basement membrane. Furthermore, in the mesonephric amphibian glomerulus there is no sub-endothelial basement membrane. It is possible that the absence of the latter is due to a physiological state of the capillary loop basement membrane, for Gersh and Catchpole (1949) consider that basement membrane is closely related to intercellular ground substance and may be merely a concentration of ground substance. They consider basement membrane to have a labile character.

Electron microscopical studies in the metanephric glomeruli of higher mammals, marsupials and birds have revealed three types of capillary loop basement membrane. Firstly, there is the three-layer type described by Pease (1955), Yamada (1955), Rhodin (1955), Saguchi (1955), Pak Poy and Robertson (1957); secondly, there is the single-layer type described by Hall (1953) and Reid (1954), while Pak Poy and Robertson (1957) published a photomicrograph showing the middle dense layer spreading over to come into contact with the endothelial cytoplasmic membrane; and thirdly, the splitting up of the middle dense layer with less dense layers on either side described by Pak Poy (1957) in the marsupial glomerulus. This is further evidence that the basement membrane is a variable structure. Finally, the difference in structure of the capillary loop basement membrane in the toad may be a Class difference, because this animal produces more urine weight for weight than mammals and the urine is hypotonic to blood.

It is interesting also to compare the mesonephric renal glomerulus to the developing metanephric glomerulus. Certainly the continuity of Bowman's capsular basement membrane with the sub-epithelial basement membrane is compatible with the invagination theory of glomerular formation. However, it is also compatible with the findings of Herring (1900), who considers that the capillaries are formed *in situ*, and of Hall and Roth (1956) who reached the same conclusions in their electron microscopic studies. Herring (1900) did not see any basement membrane but implies that there may be a Bowman's capsular basement membrane in the developing glomerulus. Hall and Roth (1956) describe a Bowman's capsular basement membrane, in the developing metanephric glomerulus and it does not seem unreasonable to suppose that in a more mature glomerulus there is a sub-epithelial basement membrane for these cells, Bowman's capsular cells and the epithelial cells, have the same origin. The similarity of a mesonephric glomerulus with its single sub-epithelial basement membrane to the developing metanephric glomerulus, is now evident.

Light microscopists have always described the capillary loop basement membrane as single. However, Jones (1957) postulated that this apparent single basement membrane was divided into its two components (sub-endothelial and sub-epithelial) by exudate in disease processes and he called the space between the two components the peri-capillary space. It was thought that if this space did exist in the normal glomerulus it may be visible in the electron microscope, but this has not been so in the metanephric glomeruli. However, in a mesonephric glomerulus there is an actual peri-capillary space containing cells, limited on one side by a sub-epithelial basement membrane and on the other by endothelial cytoplasm. This finding of peri-capillary cells is compatible with both theories of glomerular formation.

In many disease processes in the glomerulus light microscopists have described thickened capillary loop basement membrane, endothelial and epithelial proliferation and the question arises whether the glomeruli studied in the present work were diseased. The constant findings in all animals examined and in two different species make this most unlikely.

SUMMARY.

Electron microscopic study of the amphibian mesonephric renal glomerulus shows that it resembles the mammalian metanephric glomerulus except for three features: (i) In the capillary loops, the space normally occupied by basement membrane in the metanephric glomerulus, is composed of a dense sub-epithelial basement membrane and a spongy moderately dense material which fills the space between the endothelial layer and the dense sub-epithelial basement membrane; (ii) in this space (the peri-capillary space) cytoplasmic projections and cells are present; (iii) the epithelial pedicellar formation is not as complex as that seen in metanephric glomerulus.

Acknowledgments. I am indebted to Professor J. S. Robertson for advice and assistance during this study.

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RIFT VALLEY FEVER VIRUS IN MICE

VI. HISTOLOGICAL CHANGES IN THE LIVER IN RELATION TO VIRUS MULTIPLICATION

by C. A. MIMS[1]

(*Accepted for publication 7th May, 1957.*)

The histological changes in the livers of mice and other mammals infected with Rift Valley Fever (RVF) virus were carefully studied more than twenty years ago by Findlay (1931-2, 1933). At that time, RVF virus titrations were not commonly made and almost nothing was known of the adsorption and multiplication of virus during the infection. It was thought that a correlation of histological change with virus multiplication could now be made in the light of the quantitative studies reported in previous papers of this series.

It is only when very large amounts of RVF virus are inoculated that all susceptible cells in the mouse are infected *ab initio* (Mims, 1956c). When smaller doses are given, a smaller number of cells are infected initially, and the rest secondarily, in later cycles of growth. Therefore, following smaller inocula, different cells are infected at widely differing intervals after inoculation, and at any given time might be expected to show correspondingly different degrees of pathological change. Under these conditions, the temporal development of cell changes during a single cycle of growth would not readily be followed. It was assumed that a saturating inoculum, itself capable of infecting all cells from the beginning, would produce a more orderly sequence of histological changes, the differences between individual cells being due principally to variations in their reaction to infection, and to a lesser extent to slight differences in the exact time of adsorption of virus. Studies have therefore been made of the histological changes in the liver of mice following the inoculation of a saturating dose of virus.

Materials and Methods.

Virus. A strain isolated from pooled Eretmapodites mosquitoes in Bwamba County, Western Uganda (Smithburn, Haddow and Gillett, 1948) which had had about 150 mouse passages, was used. Stock virus for experiments was obtained by making a pool of serum from sick or newly dead mice which, unless otherwise stated, had been inoculated with infective serum diluted 10^{-8}. Virus harvested following such small inocula is of very high titre ($10^{8.5}$-$10^{9.5}$ LD50/0·03 ml.) and contains little or no incomplete, non-infective material (Mims, 1956d).

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Mice. The mice used were of Swiss stock, their ancestors originating from Carworth Farms, New York. They were used when from four to six weeks old.

Infectivity titrations. Serial tenfold dilutions of infective material were made in pH 7·4 buffered 0·75 p.c. bovine plasma albumin (Armour, fraction V), each dilution being inoculated intracerebrally (0·03 ml.) to five or six mice. Mice were observed for five days, and 50 p.c. endpoints then calculated from deaths by the method of Reed and Muench (1938). Deaths occurring on the first day were regarded as non-specific and were not included in calculations.

Histological technique. Material for both haematoxylin-eosin and Feulgen staining was fixed in Carnoy's fluid. Material for staining by Best's Carmine method for glycogen was fixed in alcoholic acetic-picro formalin. Normal mouse livers were included as controls, with each type of stain.

The glycogen stain was employed because it has been shown by de Burgh (1950) that a disappearance of glycogen is one of the first histologically demonstrable changes in mouse liver cells infected by ectromelia virus. Mice were given 0·1 ml. of 20 p.c. glucose intraperitoneally 15-60 minutes before sacrifice. Larger volumes of glucose were often lethal for RVF infected sick mice, although normal mice were unaffected.

EXPERIMENTAL.

Histological changes during a single cycle of growth.

When mice are inoculated with more than approximately $10^{8.0}$ LD50 of RVF virus, the inoculum itself infects all susceptible cells (Mims, 1956c). In this single cycle experiment, 40 mice were each given $10^{9.7}$ LD50 intravenously. At hourly intervals three mice were sacrificed, their pooled blood titrated, and liver samples removed for histological study. Haematoxylin-eosin, Feulgen, and Best's Carmine stains were used to follow histological changes.

1 *Hour.* Many hepatic cell nuclei were swollen and the chromatin pattern coarsened showing a tendency to margination.

2 *Hours.* The nuclear changes were more intense and nearly all hepatic cells were affected. The central portion of the nucleus was clear, and there was a thin rim of chromatin at the nuclear margin. A slight but distinct decrease in glycogen content had occurred.

3 *Hours*: An inclusion body was present in virtually every hepatic cell nucleus. These inclusion bodies were composed of particles of eosinophilic material in an unstained matrix, situated in the central portion of the nucleus with a clear zone separating them from the marginated chromatin. They were usually irregular in outline, and often connected to the nuclear membrane by strands of eosinophilic material. They were Feulgen negative, and distinct from the now slightly swollen nucleolus or nucleoli visible at the nuclear margin. No normal hepatic cell nuclei were visible, although normal nuclei were present in Kupfer cells and in cells lining bile ducts and blood vessels. These cells were clearly identifiable by their position and appearance, and at no stage in the pathological process was there evidence that they were affected. The decrease in the glycogen content of cells continued. An occasional cell containing a disintegrated nucleus was seen.

4 *Hours.* Nuclear disintegration was common. At about the time of the breakdown of the nuclear membrane the chromatin became densely pyknotic and formed globules, so that nuclear fragments were thereafter clearly identifiable. In some cells the nuclear membrane had presumably broken down recently, and the nuclear remains had the appearance of a collapsed, empty bag, which had usually, but not always, reached the pyknotic stage. In others, the fragments were irregular in shape and pyknotic, and in many cells no nucleus was visible. Cytoplasmic disintegration was presumably occurring at this time, first, because nuclei were able to escape through the cell boundary, and second, because red blood corpuscles were often seen occupying part of the cytoplasmic space. A further decrease in the glycogen content of cells was observed.

5 *Hours.* Cell disintegration was now even more marked. Haemorrhages and "blood lakes" were present. The blood lakes, which were a very prominent feature, were rounded collections of blood, often surrounding liver cells or groups of liver cells, completely breaking up lobular architecture, and giving the section a characteristic appearance. The diameter of the lakes was typically 60-120 μ. Larger, irregular collections of blood were occasionally seen, and these were clearly haemorrhagic in origin. Hepatic cell fragments (pyknotic nuclear remains, intact nuclei containing inclusion bodies, and pieces of cytoplasm) were visible lying free in haemorrhages, blood lakes and blood vessels. Many cells contained little or no glycogen.

6 *Hours.* Cell disintegration was maximal, and there was a complete absence of demonstrable glycogen. A few cells were seen in which the cytoplasm gave a strongly eosinophilic hyaline staining reaction ("Councilman"-type change). Some mice were detectably sick.

6½-10½ *Hours.* All mice were sick at 6½ hours and deaths occurred from then until 10½ hours. There was no significant difference in histological appearances between mice sick at 6 hours, and those still sick at seven or eight hours. In livers taken immediately after death, however, the number and size of the blood lakes was very much greater, whether death was at 6½ or 10½ hours. A large proportion of the space which would normally consist of liver substance was then occupied by blood. Hence livers from moribund or dead infected mice were deep red in colour and friable. There was no terminal increase in the incidence of haemorrhage, which was always of variable occurrence and frequency.

It should be emphasized that, as a result of individual variation in response to infection, the principal changes occurred at slightly different times in different mice. For instance, the liver of one of the mice sacrificed at five hours had the histological appearance of a "four hour" liver, and the time of appearance of the haemorrhages and blood lakes varied from mouse to mouse.

In the figure, the growth curve obtained from the blood titrations is shown. As would be expected, points are more irregular than when successive blood samples are taken from the same mice, but the curve nevertheless is of the "one step" type, as described by Mims (1956b). There is an initial period of adsorption

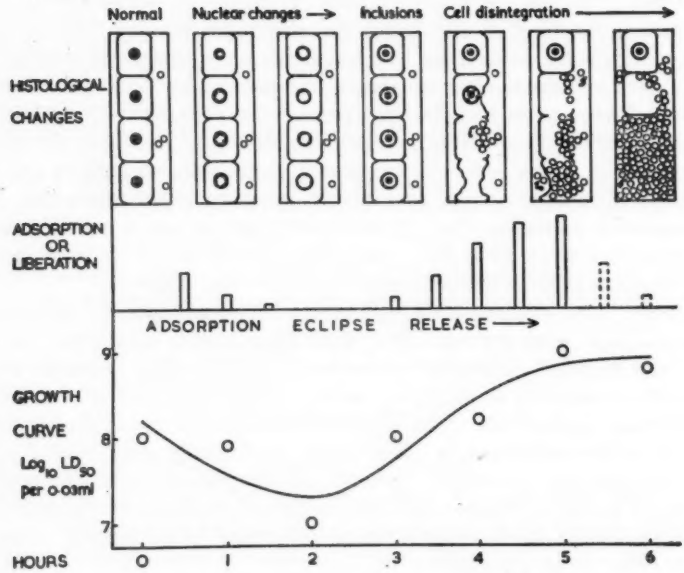

Fig. 1. Histological changes in the mouse liver during a single RVF virus growth cycle, correlated with virus behaviour. Relative amounts of virus adsorbed or liberated each half hour calculated from the growth curve and shown on an arithmetical scale. Liberation after five hours uncertain, and therefore represented by interrupted lines. The small open circles are red blood corpuscles which, in the later stages of infection, replace disintegrated cellular material and cells to form blood lakes. Degenerate nuclear fragments are shown from the fourth hour onwards.

before the virus increase is detected at about three hours, and a peak titre is reached within about six hours. The curve was drawn by eye, and from it the absolute amount of virus adsorbed or liberated in each half-hour period was calculated. These amounts are shown on an arithmetical scale, and although they are only rough estimates, it can be seen that nearly all the virus which appeared was released from 3½ to 5½ hours after inoculation. The general sequence of histological change is shown diagrammatically.

The appearance of hepatic cell fragments in the general circulation.

In the single cycle experiment, at the time when hepatic cells were observed to be disintegrating, cell fragments were seen lying free in blood vessels, blood lakes, and in haemorrhages in the liver. It was thought that these fragments probably entered the general circulation, and a search was therefore made for them in blood taken from the heart of very sick or recently dead infected mice. Blood smears were made and stained with haematoxylin and eosin, and on

several occasions typical pyknotic nuclear fragments were seen. These occurred both in mice moribund at 6 hours following very large inocula, and in mice moribund at 40 hours following very small inocula. They were not observed in smears made in the same way from normal mice, nor in infected mice before they became very sick. Although intact hepatic nuclei containing inclusion bodies could be seen lying free in blood in the liver, these were not seen in the blood smears, perhaps because they disintegrate soon after entering the circulation. Fragments of cytoplasm would be even more fragile and probably break up into very small pieces.

The significance of haemorrhages in the liver.

In the single cycle experiment haemorrhages were seen in the liver as early as five hours after the inoculation of virus. In RVF sick mice the clotting time is prolonged, and this is associated with a prothrombin deficiency (Mims, 1956e). Although this deficiency is probably due to liver damage, it is difficult to imagine the effects of this damage appearing within five hours of inoculation. An experiment was therefore carried out in which clotting times were determined from tail blood by the capillary tube method after mice had received a saturating virus inoculum. It was found that three of five sick mice tested between the fifth and the sixth hour after inoculation had prolonged clotting times. At the time the tests were made, all these mice had small liver and lung haemorrhages. Thus, even in the single cycle type of experiment, the clotting defect appears early enough to account for the haemorrhagic phenomena.

Histological changes in the livers of mice given smaller virus inocula.

The "single cycle" experiment was performed in order that each liver cell should be infected at approximately the same time. The inoculum, however, was unusually large, and it was thought possible, for reasons mentioned in the introduction to this paper, that the final histological appearance might be different when smaller inocula were given. Therefore a number of mice were inoculated intracerebrally with $10^{1.0}$, $10^{5.0}$, and $10^{7.0}$ LD50, and livers removed at 40, 30, and 23 hours respectively, when mice were moribund or just dead. Infective serum of high titre, prepared as described under materials and methods, was used for these inoculations, and four livers were examined from each inoculated group. In no case did the histological picture differ significantly from that seen 6-10 hours after the saturating inoculum in the single cycle experiment.

Histological changes in the livers of mice given inocula containing a high proportion of non-infective virus material.

As classically described by Findlay (1931-2, 1933), the livers of mice infected with RVF virus differ from those studied here. Blood lakes were not observed, and the "Councilman" type of eosinophilic hyaline change in the cytoplasm of infected cells was probably more common. The strain of virus was

of different origin from the one used in the present investigations, and this may partly account for the differences. In addition, however, Findlay's inocula probably contained incomplete, non-infective virus, for routine passages were usually made using concentrated material, and it is known that large quantities of incomplete virus accumulate under these conditions (Mims, 1956d). The effect of incomplete virus on the pathological picture produced was therefore investigated.

A passage series was started by inoculating mice intracerebrally with tenfold diluted stock high titre infective serum. When these mice were sick or recently dead, blood was obtained, pooled, and the serum inoculated as before into another group of mice. In successive passages the serum titre fell markedly, because incomplete virus accumulated (Mims, 1956d). At various stages in this passage series livers were removed from very sick or dead mice, fixed in Carnoy's fluid, and stained with haematoxylin and eosin. On examining these livers it was seen that at certain passage levels blood lakes either did not occur, or were infrequent, whereas in the large number of livers which have now been examined following inoculation with standard high titre virus, blood lakes have invariably been prominent. The "Councilman" type of cytoplasmic change was now very common, whereas it had been uncommon when standard ("complete") virus was used. Affected cells were distributed irregularly, with no apparent relation to lobular structure, and many of them consisted of a rounded lump of eosinophilic material with the nucleus situated at the edge. It was not possible to say with certainty whether cell breakdown was less extensive in the incomplete virus series. It may be inferred, therefore, that inocula containing incomplete virus do not necessarily produce blood lakes, and that the "Councilman" type changes in the cytoplasm are more common.

A further experiment was performed to see whether heat inactivated virus behaved in the same way as incomplete virus. Stock infective serum of high titre ($10^{9.1}$) was heated at 56° C. for 2 and for 3 hours, a treatment which depressed the titres to $10^{5.9}$ and $10^{4.8}$ respectively. These heated sera were then inoculated undiluted intracerebrally into groups of mice, whose livers were examined histologically in the usual way at the time of sickness and death. As with incomplete virus inocula, there was more "Councilman" type material in the cytoplasm of cells and few or no blood lakes.

DISCUSSION.

Very few studies have been made in which the histological changes in infected cells are correlated with the adsorption, multiplication, and release of virus from these cells. For investigations of this kind, a single cycle of growth has to be produced in cells, and the time relations of this single cycle known.

The RVF studies reported in the present paper are obviously less precise than those of Lwoff, Dulbecco, Vogt and Lwoff (1955), who were able to isolate

single cells *in vitro*, infect them with poliomyelitis virus, and then make parallel studies of the release of virus from and changes in these single cells. For one thing individual mice, as well as individual cells, vary somewhat in their response to infection, and a general picture of the process is seen against the background of these variations. Again, the amount of virus injected in the experiment ($10^{9.7}$ LD50) was probably about 50-100 times the amount ($10^{8.0}$ LD50) needed to infect all susceptible cells, and it is therefore possible that each hepatic cell adsorbed 50-100 times the number of particles needed to infect it. It was shown (Mims, 1956b) that virus is released earlier when larger doses are given, and in the experiment release had begun as early as 3 hours after inoculation. It may be that the histological changes are also accelerated under these circumstances. Further, RVF titration inaccuracies may be of the order of $10^{0.5}$ LD50/0·03 ml. (Mims, 1956a), and since virus released into the blood has to be detected in relation to the amount already present, the exact amounts released towards the end of the growth curve cannot be estimated very precisely. Nevertheless, from information obtained in the experiments described in this paper, the following general outline can be given of the course of events when mouse liver cells are infected with RVF virus. The behaviour of infective virus is deduced from the growth curve in accordance with the data presented by Mims (1956b).

Virus enters liver cells from the blood, and becomes non-infective almost immediately. About 75 p.c. of this adsorption takes place within thirty minutes, and the remainder within the next hour. Within an hour of inoculation, many liver cell nuclei have reacted to infection by becoming swollen with their chromatin pattern coarsened and marginated. By two hours nearly all nuclei are in this condition. At three hours the changes are more marked and, in addition, intranuclear inclusion bodies are present in all liver cells. The latent or eclipse period, when virus is presumed to be multiplying in a non-infective form intracellularly, occurs between the first and third hours. By the fourth hour, when the release of virus into the blood stream is under way, cells have begun to disintegrate. The nucleus ruptures, the cytoplasm breaks down, and as the degenerate fragments enter the circulation red blood corpuscles occupy the empty spaces. Virus continues to be released at an ever increasing rate, and at five hours nuclear and cytoplasmic disintegration is widespread. Blood lakes are now found, and haemorrhages begin to occur. It is difficult to say whether the growth curve continues to rise after the fifth hour, although quite probably large quantities of virus continue to be released until about the sixth hour. Cell disintegration and blood lakes are even more extensive at six hours, and demonstrable glycogen has by now disappeared from cells.

At 6½ hours sickness was first detectable, and although in the experiment some mice died at this stage and some not for another three hours, there were no significant histological changes during this time, except for a terminal increase in the extent of the blood lakes. It is thought possible that the blood lakes are formed by blood from sinusoids coming to occupy the spaces left by hepatic

cells which have disintegrated and disappeared. At an earlier stage red blood corpuscles would be present both in the cytoplasm of disintegrating cells, and in the spaces left by single missing cells, as was observed. The process would be assisted if the massive cell necrosis resulted in a general weakening of the liver architecture. The lakes are first seen five hours after inoculation and by this time hepatic cell breakdown is extensive and degenerate cell fragments are visible lying free in blood in the liver. The terminal increase in blood lakes is perhaps attributable to a back pressure in the hepatic veins which might be sufficient to distend and extend the lakes, especially if the damaged liver stroma was now less resistant to distortion.

The cell disintegration observed in the late stages of infection was on a large scale, even when very small amounts of virus had been inoculated, and it is probable that a correspondingly large number of degenerate cell fragments were released into the circulation. It may be noted that Cowdry and Kitchen (1930) observed degenerate hepatic cells lying free in blood vessels in the liver of Yellow Fever-infected monkeys, but the peripheral blood was not examined.

Nothing is known of the intracellular site of origin of RVF virus, nor of the way in which it is liberated from cells. Although in the single cycle experiment infective virus began to appear at about the same time as cells began to disintegrate, it cannot be concluded that liberation is necessarily by cell disintegration. It has been shown by Reissig and Melnick (1955) that herpes virus originates in the nucleus of infected cells and appears in the cytoplasm at a later stage. This was confirmed in fluorescent antibody studies by Lebrun (1956), who showed that by the time the intranuclear inclusion body was fully developed, it contained no demonstrable virus antigen, so that it could be described, as suggested by Crouse *et al.* (1950), as a mere "Morphological residuum" of infection. This could also be the case for RVF virus, and, since the intranuclear inclusion bodies are well developed by three hours, it would mean that virus material had already been largely transferred to the cytoplasm by this time.

Reissig and Melnick (1955) also showed that the herpetic intranuclear inclusion bodies are probably fixation artifacts produced by shrinking of eosinophilic material away from the marginated chromatin to the centre of the nucleus. If this were also true of RVF virus inclusion bodies, it would explain why they are often connected to the edge of the nucleus by eosinophilic strands.

A coagulation defect was shown to be present five hours after the administration of the saturating inoculum. This signifies that, at that time, prothrombin production by the liver had been depressed, and in addition that most of the circulating prothrombin had disappeared. Although liver cells showed histological evidence of damage within one hour, circulating prothrombin must disappear at a remarkably rapid rate to result in a deficiency within five hours. There is, however, a very rapid rate of turnover of metabolites in small mammals like the mouse. Dixon, Maurer and Deichmiller (1953), for instance, showed

that while the half life of homologous serum albumin was 15 and 20·7 days respectively in man and in the cow, it was only 1·2 days in the mouse.

When mice were given inocula containing large amounts of non-infective virus material, there were notable differences in liver pathology, whether the non-infective material was produced by heat inactivation, or whether it was in the form of "incomplete" virus. Blood lakes ceased to be prominent, but although it is suggested above that blood lake formation is related to an extensive cell breakdown, it was not possible to say with certainty that this breakdown was now less marked. The "Councilman" type changes in the cytoplasm were more common and since there is evidence that when the yield contains much incomplete virus there is a delay in its release from the liver (Mims, 1956d), it is conceivable that the "Councilman" type changes are associated with the retention of virus particles in the cell.

SUMMARY.

The development of histological changes in the livers of mice following the inoculation of a saturating dose of RVF virus is described.

The first detectable changes were observed in the nucleus of hepatic cells after one hour, and intranuclear inclusion bodies were present in nearly all these cells by the end of the eclipse period at about three hours.

Nearly all virus was released between 3½ and 5½ hours after inoculation and an extensive disintegration of hepatic cells accompanied this release. Large numbers of disintegrated cell fragments entered the circulation.

Blood lakes were a prominent feature, and haemorrhages occurred in association with a coagulation defect which was detectable five hours after inoculation.

There was a progressive disappearance of glycogen from hepatic cells, and none could be detected at six hours. Sickness and death began to occur at this time.

The same final histological picture was produced when mice died 40 hours after a very small virus inoculation.

When inocula contained large quantities of non-infective virus material, the histological appearance became more similar to that originally described for RVF virus by Findlay.

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THE DIRECT EFFECT OF OESTRADIOL ON THE PARS DISTALIS

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In a previous experiment we demonstrated that hydrocortisone acts directly at the pituitary level to inhibit ACTH production (Rose and Nelson, 1956a). In this paper the effects on the pituitary of castrate male rats given a continuous infusion of oestradiol directly into the hypophyseal fossa, just outside the fossa, or subcutaneously will be described. The evidence obtained from these experiments supports the theory that oestrogen acts directly at the pituitary level to influence gonadotrophic hormone production.

METHODS.

Preparation and administration of oestradiol solution. A solution containing 10 μg. per ml. of oestradiol was prepared as follows: 1 mg. of oestradiol was dissolved in 2 ml. of ethyl alcohol and added to 100 ml. of a boiling solution containing M/33 glycerine buffer at pH 10·5 and 5 p.c. glycerol. The micro-injectors delivered 0·01 to 0·04 ml. per diem.

Operative and micro-injector technique. The preparation and use of the micro-injector (Rose and Nelson, 1955), the detailed technique for the introduction and fixation of a plastic tube into or just outside the hypophyseal fossa (H.F.) and the general and endocrine results of such operative interference have been described (Rose and Nelson, 1956b). In the following experiments we used male rats weighing 200-250 gm. which had been castrated for 7 to 8 months. At this stage the plastic tube was introduced and fixed into or just outside the H.F.; 3 days later the micro-injector was buried in a subcutaneous pocket and immediately connected to the plastic tube.

Pituitary histology. The pituitaries were obtained immediately after the animals were killed at the conclusion of the experiment. They were fixed in a mixture of formalin (1 part) and saturated mercuric chloride (9 parts) for 6 hours. Serial horizontal 7 μ. sections of the pituitary were cut and stained with Crossmon's modification of Mallory's triple stain (Crossmon, 1937) or by the periodic acid Schiff process (P.A.S.) which demonstrates the glycoprotein content of the pituitary cells (Purves and Griesbach, 1951). We have adopted the criteria as set out by these authors (1951, 1954, 1955) to distinguish the normal and castrate basophils into two types. Thus, F.S.H. cells are mainly found in the periphery especially around portal blood vessels. They have coarse granules and are strongly positive in their glycoprotein reaction. The L.H. cells are central, large, have fine granules and are only weakly stained in their glycoprotein reaction.

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Experimental design. The animals were divided into 5 experimental groups. The figure in brackets shows the number of animals in that group.

Group 1. Control castrates (4).

Group 2. "Operated" control castrates. These had a plastic tube implanted into the H.F. for 17 days and an injector subcutaneously for the last 14 days of the experiment (4).

Group 3. Castrate rats receiving oestradiol benzoate in oil as a daily subcutaneous injection for 14 days. This group is subdivided as follows:

3·1 5 μg. per diem (2).
3·2 2 μg. per diem (4).
3·3 0·5 μg. per diem (2).
3·4 0·1 μg. per diem (2).

Group 4. Castrate rats receiving a continuous infusion of oestradiol just outside the H.F., i.e. just to one side of the H.F. or just outside the dura on the dorso-cephalic surface of the H.F. (3).

Group 5. Castrate rats receiving a continuous infusion of oestradiol directly into the H.F. (4).

Results and Discussion.

The pituitary changes following gonadectomy have been a subject of interest for some years. Recently Purves and Griesbach (1955) described the progressive change in the gonadotroph cell pattern of the pituitary which takes place in the 18 months following gonadectomy. The pituitaries of the animals in Group 1 show a great increase in the number of basophil cells, many of which show vesiculation or formation of "signet rings". Following the criteria of Purves and Griesbach and in agreement with their results, most of the basophils found in the pituitaries of our rats 6 to 8 months after castration are L.H. cells.

The introduction and fixation of a polythene tube into the H.F. for 17 days (Group 2) does not result in gross pituitary damage. A moderate connective tissue reaction develops around the polythene tube and small infarcts are sometimes seen. The castrate picture as far as gonadotrophs are concerned is not obviously different from those in Group 1 (Figs. 1 and 2).

The castrate changes in the pituitaries of the animals in Groups 3·1 and 3·2 are definitely present, but look dark and regressed, "signet ring" cells can still be seen (Fig. 3). The castrate changes are still very marked in the animals of Group 3·3 (Fig. 4) and completely unmodified in Group 3·4.

The pituitaries of rats receiving a continuous infusion of oestradiol outside the H.F. in a dose range of 0·1 to 0·4 μg. per diem show very marked castrate changes not differing obviously from the castrate controls.

The picture is entirely different when the oestradiol is infused directly into the H.F. At a dose range of 0·1 to 0·35 μg. per diem the castrate changes are very much reduced. This is especially true of vesiculated gonadotrophs whilst younger gonadotrophs of the castration type have virtually disappeared (Figs. 5 and 5a). In at least two cases an obvious graded response in the pituitary can be observed. The polythene tube is introduced and fixed into the anterior

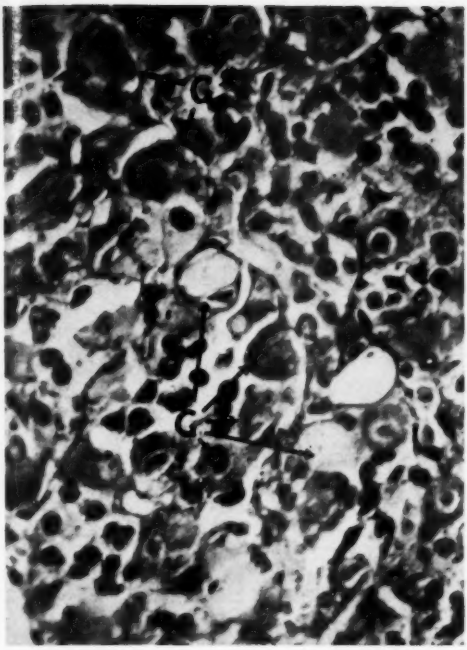

Fig. 1. Peripheral gonadotrophs of a male rat 7 months after castration. A tube has been implanted in the H.F. for 17 days. A high proportion of vesiculation and "signet ring" cells is present. (× 580.)

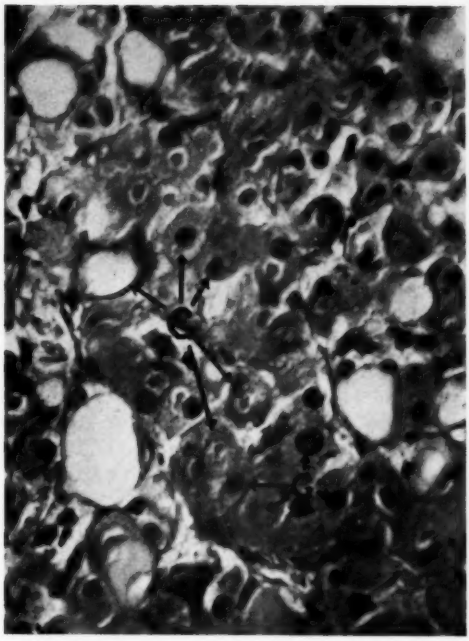

Fig. 2. Central gonadotrophs from the same rat. (× 580.)

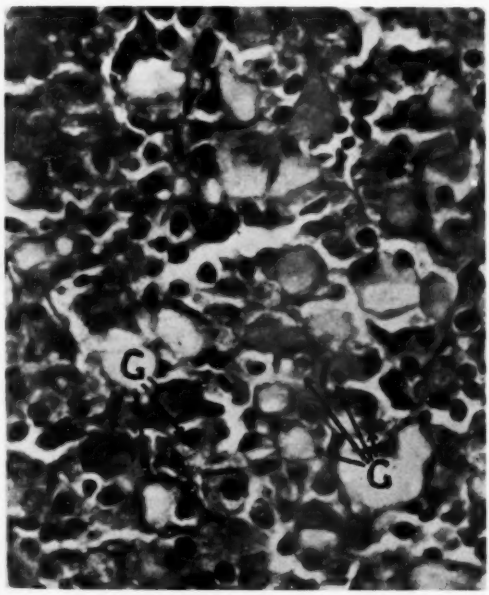

Fig. 3. Peripheral gonadotrophs of a male rat 7 months after castration and following subcutaneous injection of 2 μg. per diem of oestradiol for 14 days. Castrate changes are still present, but the gonadotrophs look dark and regressed. (× 580.)

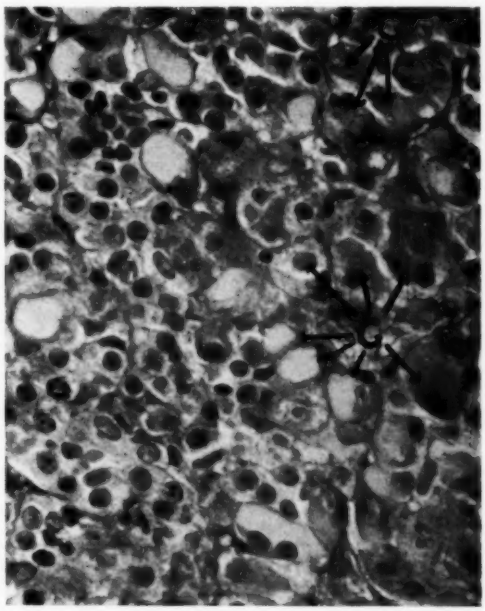

Fig. 4. Peripheral gonadotrophs of a male rat 7 months after castration. The rat received 0·5 μg. per diem of oestradiol in oil subcutaneously for 14 days. Marked castrate changes are still present. (× 580.)

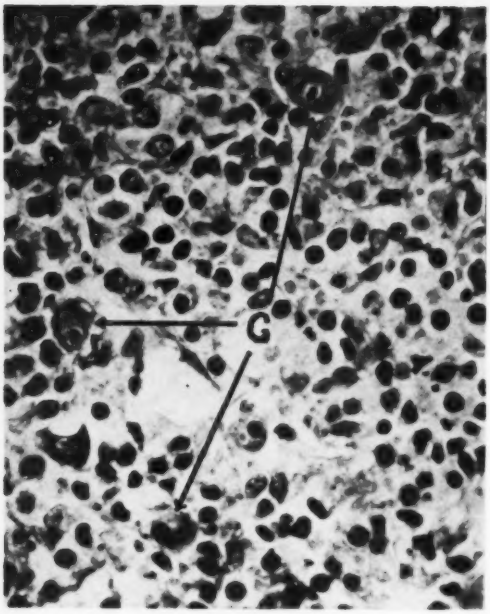

Fig. 5. Central gonadotrops of a male rat 7 months after castration, 0·32 μg. of oestradiol per diem was infused directly into the H.F. (× 850.)

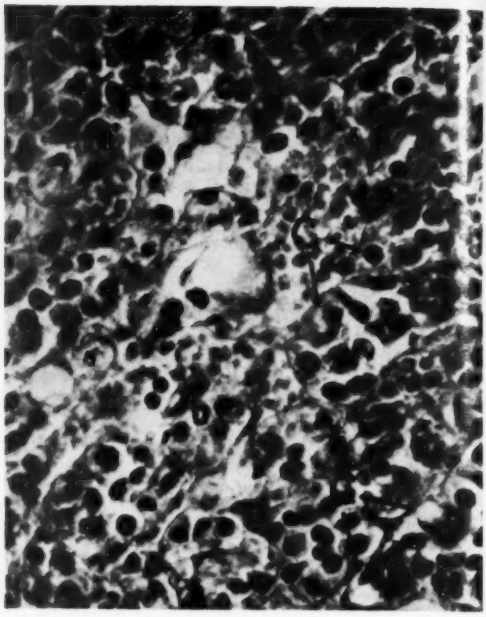

Fig. 5 (a). Peripheral gonadotrophs corresponding to Fig. 5. The castrate changes in gonadotrophs are greatly reduced, those gonadotrophs which are present are small and regressed. (× 580.)

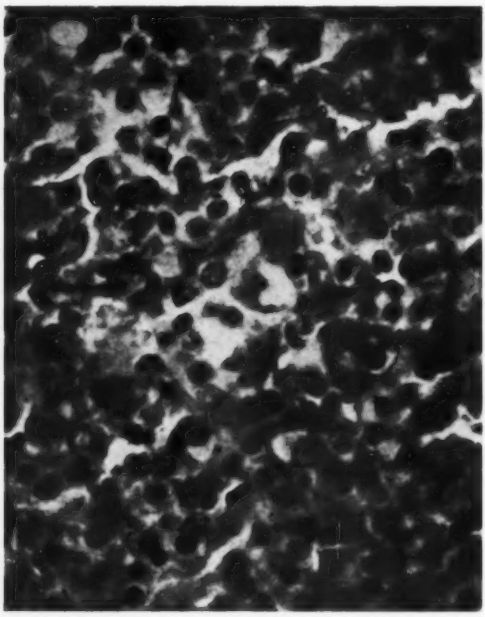

Fig. 6. Rat castrated for 7 months and receiving 0·27 μg. of oestradiol per diem into the H.F. for 14 days. Anterior edge of the pituitary showing very few gonadotrophs. (× 580.)

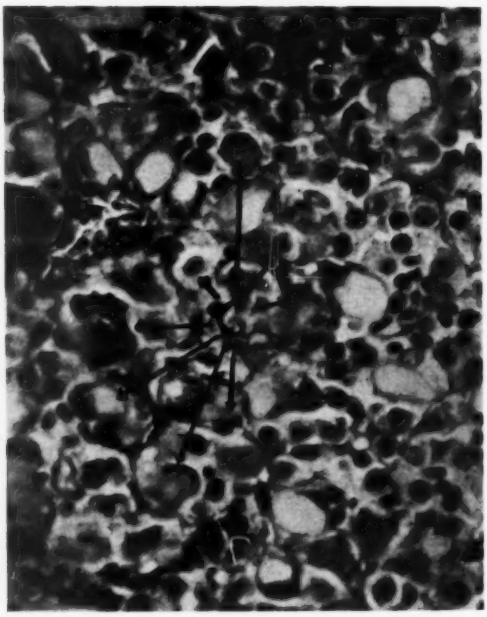

Fig. 7. Posterior edge of pituitary corresponding to Fig. 6. Castrate changes and numerous gonadotrophs may be seen. The oestradiol has produced a graded response, i.e. a marked effect on the anterior half and a lesser effect on the posterior half of the pituitary. (× 580.)

edge of the H.F. and it is in this area that the greatest inhibition of gonadotroph formation has occurred in these two cases. This is especially striking because the anterior edge usually shows the greatest number of F.S.H. cells (Purves and Griesbach, 1951). Figure 6 is a photomicrograph of the anterior edge of the pituitary of a rat receiving 0·27 μg. of oestradiol per diem directly into the anterior edge of the H.F. This shows a virtually complete disappearance of castrate and gonadotroph cells in the area. This photomicrograph should be compared with the posterior edge of the same pituitary (Fig. 7) and the anterior edge of the pituitary of a rat which received 2·0 μg. (approximately 87 times as much) of oestradiol systemically and which still shows numerous castrate cells (Fig. 3).

A pituitary of particular interest is one which received 0·3 μg. per diem directly into the anterior edge of the H.F. The castrate changes throughout the pituitary but particularly in the anterior edge have virtually disappeared. There is a small infarct which has been fibrosed in the posterior half of the pituitary and posterior to the infarct there is an area containing numerous castrate gonadotrophs. It would seem that the infarct has decreased the diffusion of oestradiol to the pituitary just behind it.

Perfusion of oestradiol into the H.F. produces a greater inhibitory effect than either, the same dose perfused just outside the H.F. and under the hypothalamus, or larger doses given systemically. These results are in agreement with the theory that oestradiol has a direct effect on the pituitary to influence gonadotrophic hormone production and release.

SUMMARY.

Using a micro-injector technique oestradiol has been infused directly (a) into the H.F., (b) just outside the H.F., or (c) subcutaneously into male rats which have been castrated for 7 to 8 months.

The infusion of oestradiol directly into but not outside the H.F. depresses the castrate changes in the gonadotrophs more markedly than 7 to 10 times the same dose given systemically.

A graded response from the anterior to the posterior edge of the pituitary is sometimes observed when oestradiol is infused into the anterior edge of the H.F.

These results support the theory that oestradiol has a direct effect on the pituitary to influence gonadotrophic hormone production and release.

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